Clinical and molecular characterization of the type I interferon signature in rheumatic diseases

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Tamarah Desirée de Jong

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Clinical and molecular characterization of the type I interferon signature in rheumatic diseases

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promotoren:	prof.dr. C.L. Verweij †
	prof.dr. J.W.J. Bijlsma
copromotoren:	dr. S. Stahlecker-Vosslamber
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Diversity is the one true thing we all have in common
(Winston Churchill)

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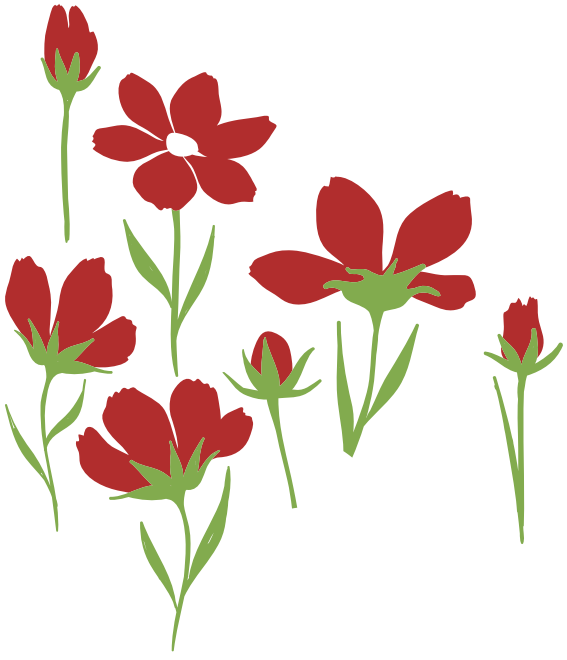
Chapter 1

General introduction



The challenge of autoimmune disease heterogeneity

The word "heterogeneous" originates from the Ancient Greek words heteros ("other") and genos ("kind") and is defined as "diverse in character or content" (Oxford Dictionary). Such diversity is observed in many autoimmune diseases; patients with the same diagnosis often exhibit differences in symptoms, disease progression and disease severity, which is thought to originate from genetic, environmental and developmental variation. An immediate consequence of this disease heterogeneity is the challenge of finding suitable treatment for every patient. Current medicine aims to beneficially treat the majority of patients, but the incidence of non-response in part of the patient population has appeared almost inevitable. As a result, one focus in research has been to develop treatment which is tailored to the individual patient, i.e. "personalized medicine". Key to achieving this is (1) to converge the diversity within diseases by identification of patient subgroups and (2) to understand the processes underlying the differences between these subgroups.

Gene expression profiling, which explores the activity of genes, has proven to be a powerful tool for subdivision of patients at the molecular level, and has also been successfully applied in the field of rheumatoid arthritis (RA) (1;2). This thesis is focused on a particular gene profile that defines a subgroup of RA patients: the type I IFN signature. We studied the potential clinical applicability of this profile and aimed to characterize its underlying molecular processes. Eventually, such studies will increase the understanding of disease heterogeneity and, ultimately, provide the knowledge to develop personalized treatment strategies.

Autoimmunity

The term autoimmunity refers to the incidence in which an immune response occurs against the body's own tissue or organs. Under normal conditions, the immune system functions by detecting and clearing pathogens in order to protect the body from disease. Under autoimmune conditions, however, the body's immune cells have lost their ability to distinguish between "self" and "non-self" and have developed an immune response towards their own body (3). There are several autoimmune diseases, often defined by the organs or tissues that are mainly targeted by the autoimmune response, such as diabetes mellitus (affecting the pancreas), Graves' disease (affecting the thyroid), rheumatoid arthritis (mainly affecting the joints), multiple sclerosis (mainly affecting the nervous system), idiopathic inflammatory myopathies (mainly affecting the muscles) and systemic lupus erythematosus (affecting several organs). Approximately 7-9% of the worldwide population suffers from an autoimmune disease (4) and for most autoimmune diseases the exact cause is unknown. In order to develop and improve therapies, to prevent damage or ultimately to prevent a disease itself, understanding the pathologies is vital.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the joints. It affects approximately 1% of the worldwide population, and women are affected twice more often than men. Symptoms involve joint pain and swelling, cartilage destruction and bone erosion. Systemic manifestations include skeletal disorders and cardiovascular complications, among others. The majority (70-90%) of RA patients display positivity for

antibodies against IgG, i.e. rheumatoid factor (RF) and/or antibodies against citrullinated proteins (ACPA).

The cause of RA is unknown, but it has been found that both RF and ACPA may be present in the patient's blood up to 14 years before disease onset (5;6), indicating that the initial trigger of autoimmunity might take place early before the actual disease develops. It has been suggested that this initial trigger could be a combination of genetic susceptibility, e.g. via variants of the HLA-DRB1 or PTPN22 genes, and environmental factors (7). The occurrence of mucosal abnormalities, such as oral infection, lung damage (e.g. due to smoking) or intestinal inflammation has been suggested as environmental factors, particularly in relation to ACPA-positive RA (8;9). At a certain point, occurrence of a certain second hit, which could be another infection, is thought to initiate the onset of actual RA, characterized by invasion of the joint synovium by immune cells, such as neutrophils, macrophages, B cells and T cells (7). Consequent production of pro-inflammatory cytokines and chemokines causes a vicious circle leading to persistence of the autoimmune response. Eventually, the pro-inflammatory cytokines induce activation and increased proliferation of fibroblasts and osteoclasts, together with inhibition of chondrocytes and osteoblasts, resulting in hyperplasia, cartilage destruction and bone erosion.

RA is known as a heterogeneous disease, as there are many differences in disease manifestations among patients. By definition, all patients suffer from joint pain and swelling, but the location and number of affected joints, as well as the severity of the damage are largely variable between patients. Also, there are differences in autoantibody positivity; patients may be autoantibody negative, single positive for RF or ACPA, or display positivity for both. Moreover, some patients show positivity for other types of autoantibodies as well (10;11). The heterogeneity of RA is also observed in the response to therapy, as reflected by the myriad of therapy options that are available for patients.

Treatment of rheumatoid arthritis

Treatment of RA consists of non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids (GCs) and disease-modifying anti-rheumatic drugs (DMARDs), both non-biologic and biologic (12). Early after diagnosis, RA patients for whom NSAID treatment is no longer sufficient are usually prescribed non-biologic DMARDs, most often methotrexate (MTX), sometimes leflunomide, sulfasalazine (SSZ) or hydroxychloroquine (HCQ). These drugs dampen the inflammation, but prolonged usage often leads to resistance. When patients no longer benefit from the non-biologic DMARD therapy, they switch to biologic DMARD therapy with a TNFα blocker. Several TNFα blockers exist, and they act by specific neutralization of the inflammatory mediator TNFα. When the inflammation persists despite anti-TNF therapy, patients switch again to treatment with another biologic DMARD, which could be rituximab, an antibody acting on the CD20 molecule on B cells, tocilizumab, acting on the receptor for IL-6, another inflammatory cytokine, or abatacept, acting on the co-stimulation molecules CD80 and CD86 on antigen presenting cells. During biologic DMARD treatment, co-treatment with non-biologic DMARDs is common (12).

GCs, such as prednisone, are agonists to the glucocorticoid receptor and are prescribed throughout the whole disease course of RA. The anti-inflammatory mechanisms are not ful-

ly understood, but they involve reduction of pro-inflammatory cytokines via repression of the transcriptional activity of the NFκB protein and pain reduction via inhibition of prostaglandins, among others (13). GCs were initially prescribed to RA patients in high doses (≥10mg/day) to suppress flares of inflammation, but nowadays long-term treatment with low-dose GCs is used more commonly (14). Often, they are applied as a bridging therapy to prevent flares in between treatments (15). Co-treatment with GCs during DMARD therapy is also common and is shown to be beneficial, at least for non-biologic DMARDs and particularly in the early phase of the disease. Several trials have demonstrated that treatment of early RA patients with a combination of prednisone with MTX, SSZ and/or HCQ results in a decrease in disease activity, functional improvement and less radiographic joint damage compared to monotherapy (16-18). The additional value of GC use during biologic therapy remains to be determined (19).

Rituximab

This thesis is particularly focused on the biologic DMARD rituximab. Rituximab is a chimeric monoclonal antibody directed against CD20, a protein that is expressed on the surface of naïve, mature and memory B cells, but not on precursor B cells or plasma cells. Binding of rituximab leads to depletion of these B cells, via three proposed mechanisms: antibody-dependent cellular cytotoxicity (initiated by recognition of rituximab by Fc receptors), complement-mediated cytotoxicity (initiated by recruitment of complement) and apoptosis (induced via intracellular CD20 signaling upon binding of rituximab) (20-22).

Rituximab treatment in rheumatoid arthritis patients consists of two injections of 500mg or 1000mg over two weeks. B cell depletion from the circulation is virtually complete already at 1 month after the first injection (mean decrease 97% (23)), but is not permanent; B cells start to repopulate after 3-6 months of therapy, with inter-individual differences in the speed of repopulation (23;24). Although rituximab is described to be efficacious at the group level, 30-50% of patients do not achieve a favorable response (25;26). Occurrence of non-response is partly explained by incomplete B cell removal (27;28) but remains elusive for a large proportion of patients.

The need for personalized medicine in rheumatoid arthritis

Similar to rituximab, treatment of RA with other biologics commonly results in 30-50% non-responders as well. Unfortunately, patients are treated for at least 6 months before non-response can be properly determined, during which unnecessary side-effects could occur, the disease progresses and damage continues. As a result, the occurrence of non-response impairs the patient's quality of life and leads to high socio-economic costs.

The current treatment approach in RA consists of so-called "trial-and-error". The course of treatment as described above (12) is partly designed in a chronological order; anti-TNF therapy was the first biologic treatment that became available for RA (29;30), and efficacy of the subsequent biologics rituximab, tocilizumab and abatacept has been weighed against the performance of the anti-TNF inhibitors or have only been tested in patients refractory to anti-TNF therapy (26;31-33). However, the heterogeneous nature of RA suggests that subgroups of patients exist that each display a specific panel of activated pathways and cell types (34-36). Consequently, each

subgroup could ultimately benefit from a specific biologic. Altogether, identification of the activated pathways in RA subgroups and/or predictors of therapy response could provide ground for stratification of patients prior to treatment, ultimately leading to an optimal treatment plan for each individual patient, i.e. "personalized medicine".

Gene expression profiling of rheumatoid arthritis: the type I interferon signature

In 2007, genome-wide gene expression analysis was performed on 35 RA patients and compared to 15 healthy controls. It appeared that many genes were differently expressed between RA patients and healthy controls, but the RA patients also displayed high inter-individual variability, reflecting the disease heterogeneity (36). This study was the first to describe that approximately 50% of RA patients display elevated expression of a certain group of genes, interferon (IFN) response genes (IRG), which is referred to as a "type I IFN signature" (36).

After this discovery, studies have been described which demonstrated that this type I IFN signature in RA has potential clinical relevance. In the preclinical phase of RA, part of arthralgia patients already display a type I IFN signature, which was shown to be associated with increased risk of developing arthritis (37;38). Moreover, presence of a type I IFN signature has been found to be associated with clinical response to rituximab therapy (39-41), tocilizumab therapy (42) and anti-TNF therapy (43).

Type I interferons

Interferons (IFNs) are cytokines that were initially described for their antiviral activities (44;45). The IFN family consists of three classes: type I, type II and type III IFNs (46). The class of type I IFNs is the largest, comprising IFNα –of which 13 subtypes consist–, IFNβ, IFNε, IFNκ and IFNω in humans. The class of type II IFNs in humans only consists of IFNγ. The class of type III IFNs is most recently identified and consists of 3 IFNλ human subtypes (46). Each class interferon is produced by different cell types and signal through different receptors, indicating that they exert different functions in the immune system (46) (**Table 1**). This thesis will be focused on the class of type I IFNs.

Type I IFNs are typically induced upon pathogen recognition by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), in particular TLR3, TLR4, TLR7 and TLR9, cytosolic RNA helicases like RIG-I and MDA5, and NOD-like receptors (47;48) (**Figure 1A**). These receptors are classically activated by binding of pathogen-associated molecular patterns, such as lipids, proteins and nucleic acids originating from pathogens like bacteria and viruses (49;50). More recently, PRRs have been found to be activated upon binding of endogenous ligands as well, the so-called damage associated molecular patterns (DAMPs), like free intracellular proteins and molecules from the extracellular matrix (51;52).

Table 1 The three human interferon classes

Interferon class	Subtypes	Production	Receptors	Signaling pathway
Type I	IFNα, IFNβ, IFNε, IFNκ, IFNω	All cell types, via PRRs	IFNAR1, IFNAR2	JAK1, TYK2, STAT1, STAT2, IRF9 ISRE + GAS sequences
Type II	IFNγ	NK cells, T cells, via lectins	IFNGR1, IFNGR2	JAK1, JAK2, STAT1 GAS sequences
Type III	IFNλ1, IFNλ2, IFNλ3	Not fully elucidated	IFNLR1, IL10R2	JAK1, TYK2, STAT1, STAT2, IRF9 ISRE + GAS sequences

IFN, interferon; PRR, Pattern Recognition Receptor; IFNAR, IFN alpha/beta Receptor; JAK, Janus Kinase; TYK, Tyrosine Kinase; STAT, Signal Transducer and Activator of Transcription; IRF, Interferon Regulatory Factor; ISRE, Interferon-Stimulated Response Element; GAS, Gamma-Activated Sequence.

Type I IFNs exert their effects by binding to the IFNalpha/beta receptors IFNAR1 and IFNAR2 and subsequent activation of the JAK-STAT pathway. After binding of IFN, the receptors dimerize and recruit Janus Kinase 1 (JAK1) and Tyrosine Kinase 2 (TYK2), respectively, followed by phosphorylation of both the kinases and the receptors. The phosphorylated proteins contain binding sites for Signal Transducer and Activator of Transcription (STAT) 1 and STAT2 (46;53;54). Activated STAT1 and STAT2 recruit IFN regulatory factor (IRF) 9 to form the IFN-stimulated gene factor 3 (ISGF3) complex, or activated STAT1 forms homodimers (55). The ISGF3 complex recognizes IFN-stimulated response elements (ISRE) on DNA, whereas the STAT1 homodimers recognize gamma-activated sequences (GAS) (**Figure 1B**). Binding leads to the induction of IFN response genes (IRGs), of which up to 1000 have been identified so far (56). These genes are highly variable in their functions, reflective of the pleiotropic effects described for type I IFNs. Functional effects of type I IFN response genes involve antiviral activity, immune modulation, antigen presentation and apoptosis (57;58). The exact occurrence of these effects depends on the circumstances and cellular context.

Type I IFN signature as a biomarker for rheumatoid arthritis

Type I IFN signature as a biomarker for rituximab non-response

In a genome-wide expression study using rituximab-starting RA patients, Raterman and colleagues showed that the type I IFN signature was the only gene profile that could distinguish between eventual responders and non-responders before start of therapy. Patients with a good response to rituximab displayed low IRG expression prior to start of treatment, whereas non-responders displayed relatively high IRG expression. A gene set of 8 IRGs was identified to have clinical utility to predict the clinical outcome of rituximab treatment, as demonstrated by an area under the Receiver Operating Characteristics (ROC) curve of 0.87 (40), which is considered very good (an AUC of 0.50 is equal to chance, an AUC of 1.00 indicates perfect distinction). Aiming for 100% specificity (i.e. all responders would be correctly classified), prediction using the 8 IRGs would result in 41% sensitivity (corresponding to correct classification of 41% of the non-responders).

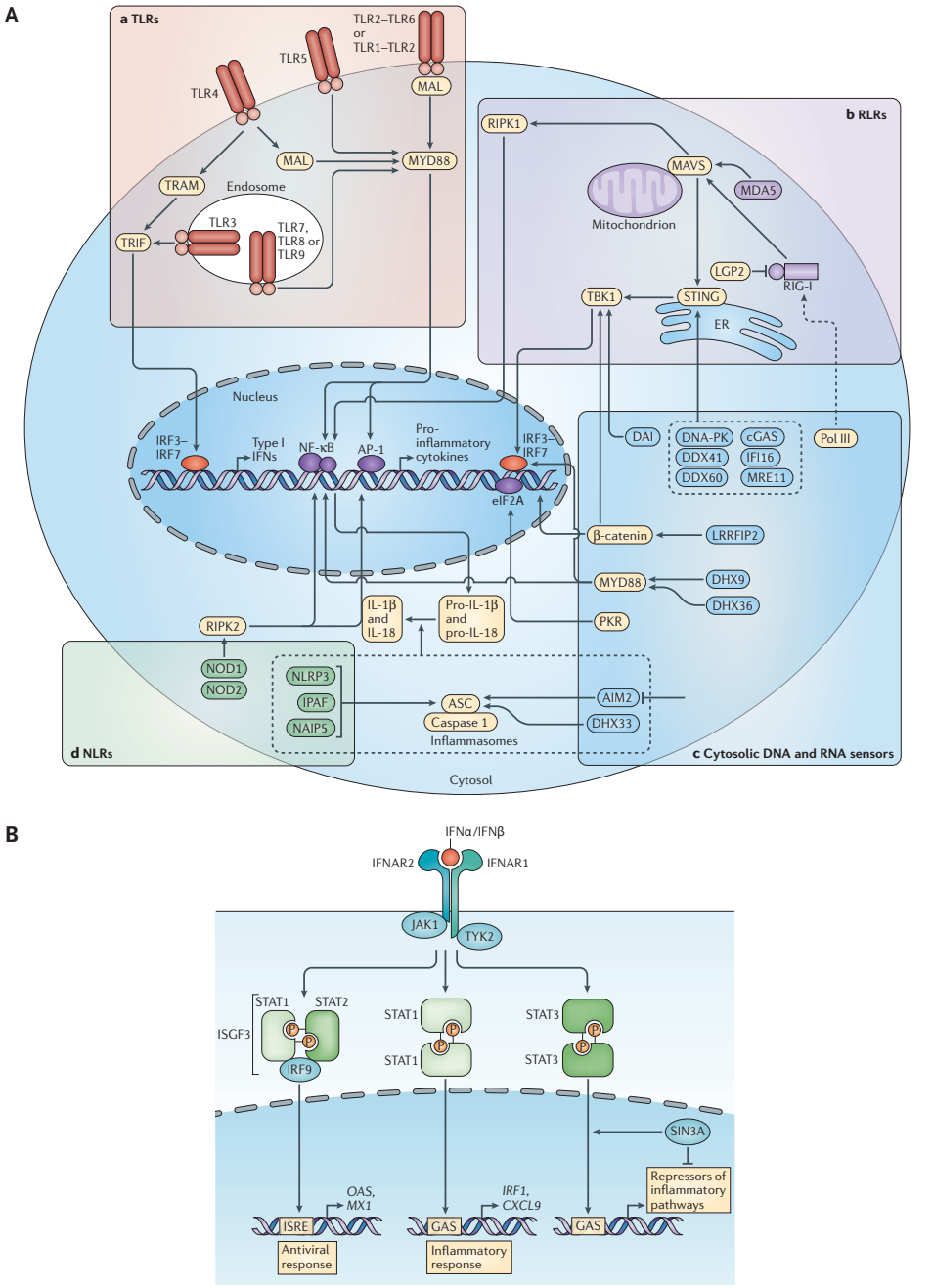


Figure 1 Signaling pathways upstream and downstream of type I IFNs. A) Signaling pathways towards induction of type I IFNs. (a) Toll like receptor (TLR) signaling (b) RIG-I-like receptor and MDA5 signaling (c) signaling by cytosolic DNA and RNA sensors (d) NOD-like receptor (NLR) signaling. Figure adapted from Cao et al.(59). B) JAK-STAT signaling pathway as activated by binding of IFNα or IFNβ to the type I IFN receptors IFNAR1 and IFNAR2. Figure from Ivashkiv et al. (55) Figures reprinted and adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology.

These findings suggest an association between IFN activity and the mechanism of rituximab in RA. Further supportive of this relation is the observation that IRG expression appeared to change during rituximab treatment, again in relation to the patient's response to therapy (41). After three months of rituximab therapy, good responders showed a temporary increase in IRG expression, which was diminished to baseline levels after 6 months of therapy. Non-responders, on the other hand, did not show any change in IRG expression. This suggests that baseline IRG expression influences rituximab response, but rituximab also influences IRG expression during therapy.

Type I IFN involvement in other RA therapies

Type I IFN response gene expression was also described to be associated to therapy response in other biologic treatments. E.g. for anti-TNF therapy with infliximab, dynamics of IRG expression appeared to be related to the clinical response, as non-responders showed an IRG upregulation during treatment whereas good responders did not (60;61). Moreover, when the type I IFN signature was determined selectively in isolated neutrophils instead of whole blood, high IRG expression at baseline appeared to be related to a good response to anti-TNF therapy with adalimumab, etanercept or golimumab (43). Furthermore, a genome-wide expression study on RA patients starting on tocilizumab, a blocker of the IL-6 receptor, revealed that high IRG expression before start of treatment is associated with a favorable response (42). The response patterns observed for these biologics are not in line with that for rituximab. Although the clinical relevance of these results need to be validated in independent studies, this may indicate that the status of the IFN system might have different clinical consequences in RA depending on the specific biologic that is used, i.e. the immune pathway that is modulated. Altogether, these findings indicate that better understanding of the mechanism behind the type I IFN signature in RA could ultimately provide insight into RA pathology and personalized treatment strategies. In order to achieve this, independent validation, determination of clinical utility and molecular characterization of the signature are necessary steps to be taken.

Type I interferons in other autoimmune diseases

Activation of the type I IFN system is not restricted to RA alone, but has been described for several autoimmune diseases, such as systemic lupus erythematosus (SLE), idiopathic inflammatory myopathies (IIM) and multiple sclerosis (MS). Most well-characterized is the role of type I IFNs in SLE, a systemic autoimmune disease in which several organs can be affected. Elevated levels of IFNα in SLE serum were already described in 1982 (62), and many times thereafter. IFNα serum levels were found to be positively correlated to disease activity in SLE and associated to involvement of certain organs, such as skin and kidneys (63;64). Moreover, SLE is characterized by the presence of antinuclear antibodies, such as anti-dsDNA and anti-U1RNP, which appeared to be associated to IFNα levels as well (64;65). It has appeared that SLE patients suffer from an impaired ability to clear apoptotic and necrotic cells, which results in enhanced exposure of intracellular proteins and DNA to the extracellular environment. Consequently, an immune response is initiated and antinuclear antibodies against these proteins and/or DNA are formed. Rönnblom and colleagues have demonstrated that immune complexes composed of antinuclear antibodies and either DNA or dead cell material are able to induce the production of IFNα via binding to Fc-gamma Receptor 2A (FcγRIIa) and TLR7 and/or TLR9, subsequently (65-68)

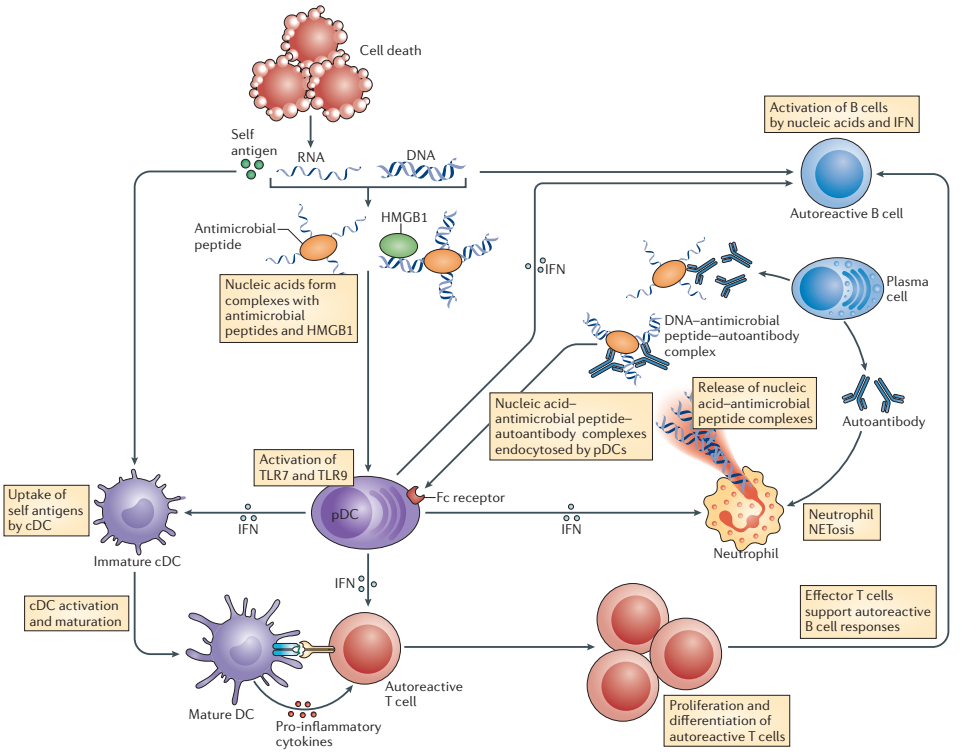


Figure 2 Mechanism of chronic type I IFN production in SLE. Figure from Ganguly et al.(79). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology.

(see **Figure 2**). The initiated immune response leads to organ damage and more apoptosis, causing a vicious circle of continuous immune activation, including type I IFN production. The increased IFNα activity in serum is reflected at the level of gene expression, as the majority of SLE patients display a type I IFN signature (69).

Similar findings have been described for IIM, an autoimmune disease mainly characterized by chronic inflammation of the muscles. Presence of a type I IFN signature has been observed in part of the patients, and was found to be associated to disease severity and disease subtypes (70;71). Like SLE patients, IIM patients often are positive for autoantibodies, such as anti-Jo1, anti-Ro52 and anti-Ro60 (72). A relation was found between the IFN signature in IIM patients and positivity for specific autoantibodies as well as positivity for multiple autoantibodies (73;74), suggesting a mechanism of IFN induction that is similar to SLE. Whether the type I IFN signature in IIM was specifically mediated by IFNα, IFNβ or another type of IFN is not yet known.

Multiple sclerosis (MS) is another autoimmune disease, in which the autoimmunity is directed against the patient's nervous system, resulting in demyelination of the nerves. In contrast to IIM and SLE, where type IFNs seem to predominantly play a pathogenic role in the disease, many

MS patients actually benefit from treatment with a type I IFN, i.e. IFNβ (75). However, a proportion of MS patients does not improve upon IFNβ therapy. Van Baarsen et al., demonstrated that part of MS patients displayed an activated pathogen-response program, including a type I IFN signature (70). In later studies, it became apparent that presence of this IFN signature before start of IFNβ therapy was related to an impaired response to IFNβ and worse clinical improvement of the patients (76;77). The exact mechanism behind the IFN signature in MS remains to be elucidated.

Mechanism of the type I IFN signature in rheumatoid arthritis

With regard to RA, It has been suggested that the type I IFN activity might be the result of a pathogen response; genome-wide expression analysis revealed that the gene expression profile in part of the RA patients resembled those of virus-infected primates (35). This patient group was mainly characterized by upregulation of TLR-related genes, which was associated with increased type I IRG expression, though not in an exclusive manner, i.e. some patients with a type I IFN signature did not show the virus-infected profile and vice versa. Other studies have demonstrated that viral infections are more common in autoimmune arthritis, including RA, compared to reactive arthritis (78), but this has not been connected to presence of an IFN signature. Altogether, the source of the IFN response in RA is yet unknown.

Thesis outline

The first part of this thesis describes studies focusing on the clinical applicability of the type I IFN signature in RA. The second part is focused on unraveling of the mechanism and source of the type I IFN signature in RA and other autoimmune diseases.

Clinical characterization of the type I IFN signature in rheumatoid arthritis

In Chapter 2 – Clinical characterization, **Chapter 2.1**, we systematically studied whether the type I IFN signature in RA was associated to clinical parameters, such as disease activity, autoantibody positivity or treatment. In **Chapter 2.2**, we focused on the effect of prednisone use on the type I IFN signature in RA and the consequences of prednisone use on the IFN-based prediction of non-response to rituximab. **Chapter 2.3** further investigated the utility of the type I IFN signature together with clinical parameters in the prediction of non-response to rituximab.

Molecular characterization of the type I IFN signature in rheumatic diseases

In Chapter 3 – Molecular characterization, **Chapter 3.1**, we studied the ability of serum from RA patients and SLE patients to induce a type I IFN response in order to gain more insight into the source of type I IFN activity in RA. In **Chapter 3.2**, we investigated the contribution of the most common immune cell types to the type I IFN signature in blood from RA patients. **Chapter 3.3** describes an in-depth study of the IFN response gene profiles that comprise the type I IFN signatures in several autoimmune diseases: SLE, MS, IIM and RA.

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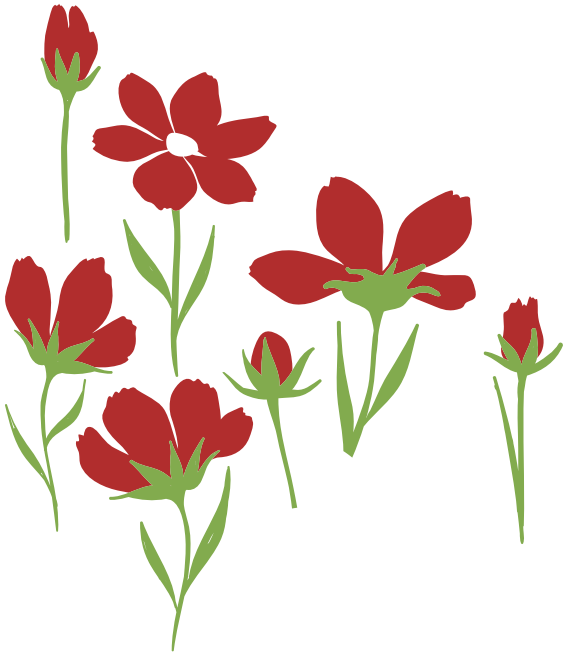
Chapter 2

Clinical characterization of the type I interferon signature in rheumatoid arthritis



Chapter 2.1

The type I interferon signature in established rheumatoid arthritis is not associated with clinical parameters

Tamarah D. de Jong*,
Marjolein Blits*,
Sander de Ridder,
Saskia Vosslamber,
Gertjan Wolbink,
Mike T. Nurmohamed,
Cornelis L. Verweij

* both authors contributed equally

Submitted for publication

Abstract

Objectives

A peripheral blood IFN signature, i.e. elevated type I IFN response gene (IRG) expression, has been described in a subset of rheumatoid arthritis (RA) patients. In the present study, we systematically assessed the association between this IFN signature and clinical parameters.

Methods

Expression of 19 IRGs was determined in peripheral blood from 182 consecutive RA patients, and averaged into an IFN score per individual. An algorithm was used to 1000 times randomize the patient group into two equally sized sets and perform correlation and unpaired comparison analysis on both.

Results

Associations were assessed between IFN score and disease duration, DAS28 and its components, the occurrence of erosions and nodules, autoantibody positivity and immunosuppressive treatment. This revealed lower IFN scores in patients using hydroxychloroquine, prednisone and/or sulfasalazine, but did not show significant associations between the other parameters and the IFN score. Selecting patients that were not treated with hydroxychloroquine, prednisone and/or sulfasalazine (n=95) did not reveal any significant associations either.

Conclusions

The IFN signature in RA is affected by immunosuppressive treatment with prednisone, hydroxychloroquine and/or sulfasalazine, but is not evidently associated with other clinical parameters. Hence, the IFN signature describes a subgroup of RA but does not reflect disease activity.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammation. It manifests itself as a heterogeneous disease, which is partly reflected at the level of gene expression. Genome-wide gene expression analysis revealed evidence for molecular differences between RA patients, in particular in the type I interferon (IFN) system. Approximately 50% of rheumatoid arthritis patients displays a peripheral blood IFN signature, i.e. relatively high expression of type IFN response genes (IRGs) (1).

Type I IFNs were initially known for their antiviral effects but increasing insight in their activities revealed their role as pleiotropic cytokines with a critical role in modulating immune responses, such as cellular activation, upregulation of the major histocompatibility complex, induction of apoptosis and inhibition of angiogenesis (2;3). It is thought that type I IFNs contribute to autoimmunity by initiating a break of tolerance, e.g. by the induction of dendritic cell maturation and inhibition of regulatory T cells (4;5). The exact role of the IFN signature in RA is yet unknown, although it was shown to have potential clinical relevance. That is, (1) the presence of the IFN signature was shown to be a risk factor for arthritis development in preclinical disease (6;7), and (2) presence of the IFN signature in established RA was found to be associated with the clinical response to treatment with rituximab (8;9) and tocilizumab (10).

Earlier studies have addressed whether the IFN signature in RA could be associated with clinical parameters, which inconclusively revealed a potential relation of the IFN signature with ACPA titers (11;12). However, these study cohorts were rather small (35 subjects or less) and therefore highly subject to a lack of power. Hence, the relation between the IFN signature and disease- and inflammation-related clinical parameters has never been thoroughly assessed. In the present study, we used a larger cohort of established RA patients (n=182) in combination with a random sampling algorithm to systematically investigate whether the IFN signature in RA could be associated with parameters such as disease activity, laboratory parameters and the use of immunosuppressive treatment.

Methods

Patient recruitment and blood collection

RA patients (n=182) were consecutively recruited from the Jan van Breemen Research Institute | Reade, Amsterdam, the Netherlands. All patients had active RA according to the ACR 1987 criteria (13) and a 28-joint Disease Activity Score (DAS28) of ≥3.2 despite earlier treatment with at least 2 DMARDs. All patients provided written informed consent and this study was approved by the medical ethics committee of Reade. Patient characteristics are shown in **Table 1**. From all patients, 2.5 ml blood was drawn in PAXgene tubes (PreAnalytix GmbH, Hombrechtikon, Switzerland) and stored at -20°C until further processing.

Table 1 Patient characteristics

	All patients (n=182)	
Demographic parameters		
Age in years, mean (SD)	54.2	(11.8)
Female, n (%)	135	(75)
Disease parameters		
Disease duration in years, mean (SD)	9.7	(10.3)
DAS28, mean (SD)	5.1	(1.2)
Erosive disease, n (%)	131	(72)
Nodules, n (%)*	43	(24)
Laboratory parameters		
ESR, mean (SD)	24.5	(18.0)
CRP, mean (SD)	17.8	(22.1)
IgM-RF titer, mean (SD)‡	124.7	(279)
IgM-RF positive, n (%)‡	95	(59)
ACPA titer, mean (SD)†	1563	(2680)
ACPA positive, n (%)†	131	(75)
Medication parameters		
MTX use, n (%)	152	(84)
MTX dosage in mg/week, mean (SD)	21.0	(6.3)
Prednisone use, n (%)	52	(29)
Prednisone dosage in mg/day, mean (SD)	7.2	(3.5)
HCQ use, n (%)	35	(19)
SSZ use, n (%)	27	(15)

ACPA, Anti-Citrullinated Protein Antibodies; CRP, C-reactive Protein; DAS28, 28-joints disease activity score; ESR, Erythrocyte Sedimentation Rate; HCQ, hydroxychloroquine; SSZ, sulfasalazine; IgM-RF, IgM Rheumatoid Factor; MTX, Methotrexate; SD, standard deviation
**Not available for 6 patients, †Not available for 7 patients. ‡Not available for 21 patients*

RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated from the PAXgene tubes according to the manufacturer's instructions. 0.25 µg RNA was reverse-transcribed into cDNA using a Revertaid H-minus cDNA synthesis kit (ThermoFisher, Waltham, MA, USA). A single aliquot of each cDNA sample was first subjected to 14 cycles of Specific Target Amplification using a 0.2X mixture of all Taqman Gene Expression assays in combination with the Taqman PreAmp Master Mix (Applied Biosystems, Foster City, CA, USA). Following pre-amplification, the samples were diluted 1:5 (v/v) in Tris-EDTA buffer, pH 8.0. Multiplex real-time qPCR was performed using the 96.96 Biomark Dynamic Array systems (Fluidigm Corporation, San Francisco, CA, USA) at ServiceXS (Leiden, The Netherlands), according to the manufacturers' instructions. Relative quantities were calculated using the standard curve method, using GAPDH as a housekeeping gene. Expression levels were $\log_2$-transformed.

Calculation of the IFN score and statistical analyses

Nineteen IRGs, described to be components of the IFN signature in RA (1), were measured (see **Supplementary Table 1**). Expression levels of the IRGs were highly correlative ($r \geq 0.7$ for 90% of the combinations, $p \leq 0.002$), therefore an IFN score was calculated by averaging the expression levels of all genes for each sample.

Data were analyzed using R version 3.1.3 (14) and GraphPad Prism 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA). To minimize finding results by chance, a 1000-times random sampling method was used to randomize the group of 182 patients into two equally sized sets and to execute Spearman correlation for continuous variables and Mann-Whitney U analysis for dichotomous variables on each set (15). P-values <0.05 were considered to be significant.

Results

We studied the association between the IFN score and the following parameters: disease duration, DAS28 and its individual components, the occurrence of erosions and nodules, autoantibody positivity and immunosuppressive treatment. As demonstrated in **Table 2**, hydroxychloroquine (HCQ) use was the only variable that showed a significant result, indicating a difference in IFN score between HCQ-treated and –untreated patients; a p value below 0.05 was detected in both sets in 521 of the 1000 iterations, in one of the two sets for 479/1000 iterations and never in none of the sets (median p value 0.015). A trend towards significance was also observed for prednisone use and dose and sulfasalazine (SSZ) use (median p values 0.090-0.14, median coefficient prednisone dose -0.18), although for these variables significance was never found in both sets. For all three treatments, the IFN score was lower in the treated group compared to the untreated group. Combining HCQ use, prednisone use and SSZ use also revealed a significantly lower IFN score in patients using HCQ and/or prednisone and/or SSZ compared to patients not treated with any of these agents (median p value ≤0.012, see **Table 2** and **Figure 1A**). Moreover, the suppressive effect appeared larger for patients treated with two or more of those agents than for patients treated with one agent (**Figure 1B**). No association was found between IFN score and MTX treatment or dose.

Since the suppression of IFN score in HCQ-, prednisone-, and/or SSZ-treated patients could have a masking effect on other associations between IFN signature and clinical parameters, we also performed the analyses for the selection of patients that were not treated with prednisone, HCQ and/or SSZ (n=95). This did not result in any significant associations between the IFN score and other variables (median p value≥0.23, see **Supplementary Table 2**).

Table 2 Analysis results of associations between IFN score and clinical parameters after 1000 times of random sampling

	Significant results (p<0.05)			Median p values	
	Both sets	One set	Neither set	Set 1	Set 2
Disease parameters					
Disease duration	0	371	629	0.18	0.20
DAS28	0	199	801	0.38	0.32
TJC28	0	264	736	0.25	0.25
SJC28	1	39	960	0.53	0.59
VAS	0	122	878	0.39	0.35
Erosions	0	61	939	0.50	0.51
Nodules	1	143	856	0.39	0.41
Laboratory parameters					
ESR	6	18	976	0.60	0.58
ESR dichotomous (>20)	0	19	981	0.60	0.58
CRP	0	233	767	0.30	0.29
CRP dichotomous (>10)	0	190	810	0.30	0.33
RF titer	1	66	933	0.46	0.50
RF positivity	3	3	994	0.62	0.61
ACPA titer	0	64	936	0.47	0.51
ACPA positivity	1	9	990	0.64	0.64
ACPA high positivity (≥3x cutoff)	0	79	921	0.44	0.44
RF and ACPA positive vs. rest	0	34	966	0.59	0.58
RF and ACPA negative vs. rest	0	219	781	0.27	0.28
Medication parameters					
MTX use	2	3	995	0.66	0.65
MTX dosage	1	29	970	0.58	0.58
Prednisone use	0	526	474	0.14	0.14
Prednisone dosage	0	718	282	0.092	0.090
HCQ use	521	479	0	0.015	0.015
SSZ use	0	584	416	0.11	0.11
PREDN and/or HCQ and/or SSZ use	612	388	0	0.012	0.010

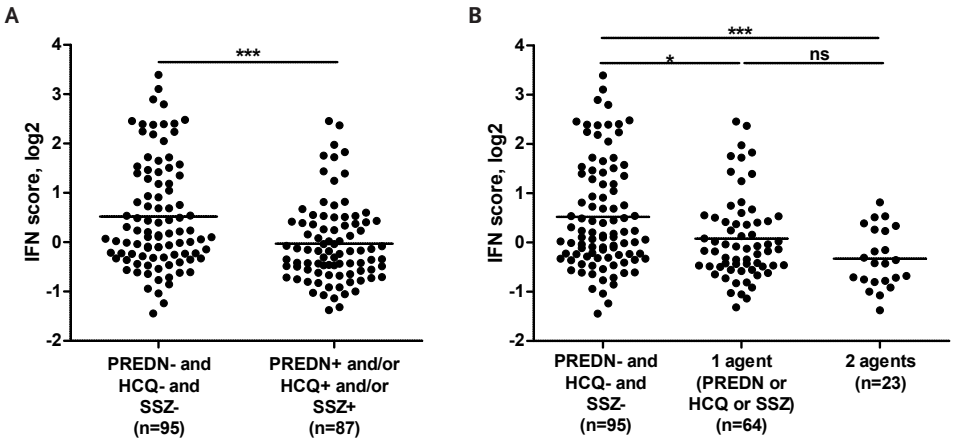


Figure 1 Comparison of IFN scores between patients not treated with prednisone (PREDN), hydroxychloroquine (HCQ) and sulfasalazine (SSZ) and patients treated with one or more of those agents. Data is displayed from the complete cohort (n=182). A) Patients divided into treated or not with one or more of the three agents; B) Patients subdivided into treated with none of the three agents, one of the three agents or two or more of the three agents. *p≤0.05, **p≤0.01, ***p≤0.001.

Discussion

The present study is the first systematic approach in a relatively large cohort to study the relation between the IFN signature in established RA and clinical parameters. We demonstrated that the IFN signature was suppressed in patients treated with HCQ, prednisone and/or SSZ, but not with MTX. Furthermore, we did not observe any associations between the IFN signature and the other clinical parameters.

Van der Pouw Kraan et al. showed that a subgroup of RA patients displays a common pathogen-response program, which was characterized by a higher incidence of the IFN signature as well as higher ACPA titers, suggesting that these parameters might be associated with one another (11). However, a causal relationship was not established, and our data now indicate that this is not the case. The IFN signature was not significantly different between ACPA-negative and ACPA-positive patients, nor did it significantly correlate with ACPA titers. Possibly, the IFN signature and ACPA positivity are independently associated with activation of the common pathogen response program, as they are both implied to be induced via certain pathogens (16;17).

Our cohort consisted of established RA patients with high disease activity (≥3.2) despite treatment with at least two DMARDs. Remarkably, the IFN scores were decreased in HCQ-, prednisone- and/or SSZ-treated patients, even though the beneficial effects of these treatments were supposedly diminished. Moreover, co-treatment with these agents appeared to have an additive suppressive effect.

Interference of both prednisone and HCQ with type I IFN signaling has been described before (18;19), but the influence of SSZ remains to be elucidated. It has been shown that SSZ reduces

the levels of RA-related cytokines, such as IL1β and TNFα (20), suggesting that SSZ might function through overall suppression of inflammatory cytokines, including type I IFNs. Furthermore, it was demonstrated that SSZ is able to accelerate apoptosis of neutrophils (21), which we have recently shown to be major inducers of the type I IFN response in RA (de Jong et al., submitted). Consequently, suppression of the type I IFN response by SSZ might be mediated via an increase in neutrophil apoptosis.

As previously described, suppression of the IFN score by certain treatment could affect the applicability of the IFN signature as a biomarker for therapy response, particularly to rituximab (9;22). That is, the treatment-related suppression of IFN score might impair the discriminative capacity of the biomarker and consequently lead to more false predictions. Future studies should elucidate the effect of each individual treatment, as well as combinatory therapy, on the IFN signature and the corresponding response prediction. Alternatively, presence of the IFN signature in individuals with arthralgia was shown to be associated with a higher risk to develop arthritis (7). It would be interesting to study whether early treatment with one of the implied suppressors of the IFN response could delay or even prevent disease onset.

In conclusion, we have demonstrated that there are no evident associations between the IFN signature in established RA and clinical parameters. This suggests that the IFN signature is not an indication of disease activity per se, but its presence could indicate a potential difference in pathology or immune pathway activation compared to patients without this signature. Consequently, this could influence the response to therapy, particularly biologics, as these are specific modulators of these immune pathways.

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Supplementary data

Supplementary Table 1 List of the 19 IFN response genes measured

Gene Symbol	Gene Name
EPSTI1	epithelial stromal interaction 1
HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5
IFI35	interferon-induced protein 35
IFI44	interferon-induced protein 44
IFI44L	interferon-induced protein 44-like
IFI6	interferon, alpha-inducible protein 6
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
IFITM1	interferon induced transmembrane protein 1
IL1RN	interleukin 1 receptor antagonist
ISG15	ISG15 ubiquitin-like modifier
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein
LY6E	lymphocyte antigen 6 complex, locus E
MX1	MX dynamin-like GTPase 1
MX2	MX dynamin-like GTPase 2
OAS1	2'-5'-oligoadenylate synthetase 1, 40/46kDa
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa
RSAD2	radical S-adenosyl methionine domain containing 2
SAMD9L	sterile alpha motif domain containing 9-like
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1

Supplementary Table 2 Analysis results of associations between IFN score and clinical parameters after 1000 times of random sampling using only patients that were not treated with prednisone, HCQ or SSZ.

	Significant results (p<0.05)			Median p values	
	Both sets	One set	Neither set	Set 1	Set 2
Disease parameters					
Disease duration	2	13	985	0.61	0.59
DAS28	0	122	878	0.42	0.46
TJC28	0	256	744	0.23	0.27
SJC28	12	15	973	0.62	0.61
VAS	4	27	969	0.57	0.55
Erosions	0	107	893	0.43	0.40
Nodules	1	129	870	0.40	0.39
Laboratory parameters					
ESR	4	19	977	0.59	0.58
ESR dichotomous (>20)	2	14	984	0.60	0.59
CRP	0	41	959	0.56	0.55
CRP dichotomous (>10)	2	8	990	0.60	0.59
RF titer	2	49	949	0.54	0.55
RF positivity	1	30	969	0.59	0.59
ACPA titer	2	27	971	0.59	0.59
ACPA positivity	0	12	988	0.62	0.62
ACPA high positivity (≥3x cutoff)	0	126	874	0.33	0.39
RF and ACPA positive vs. rest	3	18	979	0.63	0.64
RF and ACPA negative vs. rest	0	53	947	0.47	0.48
Medication parameters					
MTX use	1	4	995	0.66	0.65
MTX dosage	1	20	979	0.60	0.60

Chapter 2.2

Effect of prednisone on type I interferon signature in rheumatoid arthritis: consequences for response prediction to rituximab

Tamarah D. de Jong,
Saskia Vosslamber,
Marjolein Blits,
Gertjan Wolbink,
Mike T. Nurmohamed,
Conny J. van der Laken,
Gerrit Jansen,
Alexandre E. Voskuyl,
Cornelis L. Verweij

Arthritis Res Ther. 2015 Mar 23;17:78

Abstract

Introduction

Elevated type I interferon (IFN) response gene (IRG) expression has proven clinical relevance in predicting rituximab non-response in rheumatoid arthritis (RA). Interference between glucocorticoids (GCs) and type I IFN signaling has been demonstrated in vitro. Since GC use and dose are highly variable among patients before rituximab treatment, we aimed to determine the effect of GC use on IRG expression in relation to rituximab response prediction in RA.

Methods

In two independent cohorts of 32 and 182 biologic-free RA patients and a third cohort of 40 rituximab-starting RA patients, peripheral blood expression of selected IRGs was determined by microarray or qPCR, and an IFN-score was calculated. The baseline IFN-score was tested for its predictive value towards rituximab response in relation to GC use using Receiver Operating Characteristics (ROC) analysis in the rituximab cohort. Patients with ΔDAS28>1.2 after 6 months of rituximab were considered responders.

Results

We consistently observed suppression of IFN-score in prednisone users ($PREDN^+$) compared to non-users ($PREDN^-$). In the rituximab cohort, analysis on $PREDN^-$ patients (n=13) alone revealed improved prediction of rituximab non-response based on baseline IFN-score, with an AUC of 0.975 compared to 0.848 in all patients (n=40). Using a group-specific IFN-score-cutoff for all patients and $PREDN^-$ patients alone, sensitivity increased from 41% to 88%, respectively, combined with 100% specificity.

Conclusions

Because of prednisone-related suppression of IFN-score, higher accuracy of rituximab response prediction was achieved in $PREDN^-$ patients. These results suggest that the IFN-score-based rituximab response prediction model could be improved upon implementation of prednisone use.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic joint inflammation which may lead to cartilage and bone destruction. It is a heterogeneous disease, as reflected by differences in severity, pathogenesis and treatment outcome. From diagnosis onwards, RA patients often receive immunosuppressive treatment with non-biologic disease-modifying anti-rheumatic drugs (DMARDs) and/or glucocorticoids (GCs). When patients no longer benefit from the non-biologic therapy, they usually start on treatment with biologics, such as TNFα-blockers and B-cell depletion therapy using rituximab (RTX) (1). Approximately 30-50% of patients do not achieve a favorable response to biologics. To increase treatment efficacy and to develop personalized treatment, predictors of therapy response are needed.

Independent studies have shown that activation of the type I interferon (IFN) system is associated with the clinical outcome of rituximab therapy (2;3). This so-called "IFN signature" represents a response program consisting of genes that are activated by type I IFNs and is present in approximately 50% of RA patients (4). Induction of type I IFN response genes (IRG) is triggered via activation of the JAK-STAT signaling pathway, more specifically via JAK1, TYK2, STAT1 and STAT2, followed by recruitment of IRF9 and formation of the ISGF3 transcription factor complex (5). It was shown that patients with a good response to rituximab have low IRG expression prior to start of treatment, whereas non-responders display relatively high IRG expression. Potential clinical utility of IRG expression reflected as an IFN-score to predict the clinical outcome of rituximab treatment was demonstrated by an area under the Receiver Operating Characteristics (ROC) curve of 87% (3). Hence, knowledge on IRG expression in a RA patient before start of rituximab treatment is of crucial importance to predict the success of the clinical outcome.

It has been reported that GCs can interfere with the type I IFN system by modulation of IFN induction as well as downstream IFN signaling (6;7). GCs were initially prescribed to RA patients in high doses (≥10mg/day) to suppress flares of inflammation, but nowadays long-term treatment with low-dose GCs is commonly used as well (8). Since use and dose of GCs are highly variable among patients prior to the start of treatment with rituximab (2;3), we aimed to determine what the effect of GC use is on IRG expression in relation to the clinical response to rituximab.

Methods

Patient and controls

This study consisted of three independently collected cohorts. All patients fulfilled the revised ACR 1987 criteria for RA diagnosis (9). Patient characteristics are shown in Table 1. Cohort I included 32 RA patients of whom 6 patients were treated with the GC prednisone, as previously reported (4). Cohort II was recruited from Jan van Breemen Research Institute | Reade, Amsterdam, the Netherlands and consisted of 182 RA patients, of whom 52 patients received prednisone. The patients in these two cohorts had not been on any biologic treatment. Cohort III was recruited from Reade|Jan van Breemen Institute and VU University Medical Center, Amsterdam, the Netherlands and consisted of 40 RA patients, of whom 27 patients were using prednisone (3). These 40 patients were candidates for rituximab therapy because of their high disease activity (DAS28>3.2) despite DMARD treatment and previous anti-TNF therapy. At the

moment of blood collection, patients were off anti-TNF therapy for at least four weeks and had not received their first RTX dose yet. The clinical response to RTX was determined based on the change in DAS28 after 6 months of therapy; patients with a ΔDAS28>1.2 were considered responders (10). All patients provided written informed consent and the participating clinics received approval by the local medical ethics committee.

RNA isolation

Blood was collected in a PAXgene tube (PreAnalytix, Hombrechtikon, Switzerland) and frozen at -20°C until RNA isolation. Total RNA was isolated using the PAXgene blood RNA isolation kit according to the manufacturer's protocol. RNA quantity and purity was determined using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Delaware USA). In case of subsequent quantitative (q)PCR measurements, RNA was converted to cDNA using the Revertaid H-minus cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany), according to the manufacturer's protocol.

Gene expression measurements and calculation of the IFN-score

The IFN-score was calculated as the mean of the $\log_2$-transformed expression values of a set of IRGs for an individual patient. A set of 8 correlative IRGs (EPSTI1, HERC5, IFI44L, ISG15, LY6E, MX1, MX2 and RSAD2), previously shown to be predictive for the response to rituximab (3), was measured, unless indicated otherwise. IRG expression levels were determined by DNA microarray, multiplex qPCR (Fluidigm, Corporation, San Francisco, USA) or conventional qPCR (ABI Prism 7900HT, Applied Biosystems, Foster City, USA). To combine microarray and qPCR data, data were median-centered as described before (3).

Statistical analysis

Based on data normality, comparison of two groups was performed using the Student's unpaired t-test or Mann-Whitney U test. Comparison of multiple groups was performed using one-way ANOVA, Kruskal-Wallis one-way analysis of variance or $\chi 2$-test, where appropriate. Receiver Operating Characteristics (ROC) analyses were performed using non-responder status defined as ΔDAS28<1.2 as the state variable. All analyses were performed using IBM SPSS Statistics version 20.0 (IBMCorp, Armonk, New York). P values < 0.05 were considered to be significant.

Results

Patient characteristics

Demographic and clinical data are shown in Table 1. Whereas cohort I and II were not significantly different except for age (p=0.024)., there was a significant increase in disease activity, prednisone use and prednisone dose in cohort III (Comparison of all three cohorts, p=0.007, p<0.001 and p<0.001 respectively), probably illustrating the more severe disease state in these patients, as expected. MTX use was lower in cohort III compared to cohorts I and II. No significant differences in clinical parameters were observed in any of the cohorts between prednisone users and prednisone non-users.

Table 1 Patient characteristics of the included cohorts

	Cohort I n=32	Cohort II n=182	Cohort III (rituximab) n=40
Demographics			
Age, years	49 ± 10	54 ± 12	57 ± 10
Female, n (%)	22 (69)	135 (75)	34 (85)
Disease characteristics			
Disease duration, years	7.5 ± 8.9	9.5 ± 10.2	11.0 ± 9.5
Disease activity (DAS28)	5.4 ± 1.3	5.1 ± 1.2	5.8 ± 1.1
ESR, mm/h	29.3 ± 22.2	24.5 ± 18.0	29.2 ± 23.8
CRP, mg/l	18.8 ± 19.4†	17.8 ± 22.1	17.7 ± 17.7
Erosions, n (%)	24 (75)	131 (72)	28 (72)
IgM RF positive, n (%)	28 (88)	130 (71)	27 (68)
ACPA positive, n (%)	26 (87) ‡	129 (75)§	29 (73)
Medication			
Current prednisone use, n (%)	6 (19)	52 (29)	27 (68)
Prednisone dosage, mg/day	8 ± 2	7.2 ± 3.5	6.75 ± 6.0
Current MTX use, n (%)	25 (78)	152 (84)	26 (65)
MTX dosage, mg/week	21.2 ± 7.1	21.0 ± 6.3	18.7 ± 8.2
Current SSZ use, n (%)	N/A	27 (16)#	7 (18)
Current HCQ use, n (%)	N/A	35 (20)#	5 (13)

Continuous variables are presented as mean with standard deviation. ACPA, anti-cyclic citrullinated protein antibody; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HCQ, hydroxychloroquine; MTX, methotrexate; RF, rheumatoid factor; SSZ, sulphasalazine. N/A = not applicable. †Data missing for 7 of the 32 patients; ‡Data missing for 2 of the 32 patients; §Data missing for 9 of the 182 patients; #Data missing for 9 of the 182 patients.

Prednisone treatment and type I IFN response gene expression

To evaluate whether prednisone use affects the type I IFN-score in RA, we initially tested the relation between prednisone use and the IFN-score in patients of cohort I. Thereto, we assessed IRG expression from available microarray data (4). Since the HERC5 gene was not available on the microarray at that time, the IFN-score was based on 7 IRGs. This analysis revealed a difference between the IFN-score and prednisone use; the IFN-score was lower in $PREDN^+$ patients compared to $PREDN^-$ patients (p=0.053, **Figure 1**).

To validate the findings from cohort I, we compared the IFN-scores between $PREDN^-$ and $PREDN^+$ patients in an independent cohort consisting of 182 RA patients (cohort II). This confirmed our earlier findings, showing a significantly lower IFN-score in $PREDN^+$ patients compared to $PREDN^-$ patients (p=0.028, see **Figure 2A**). Overall, these findings reveal a relation between prednisone use and a low IFN-score.

Besides variation in prednisone use itself, the dose of prednisone varied between users, from 2.5 mg/day to 20 mg/day. Therefore, we also compared prednisone dose and IFN-score. As shown in **Figure 2B**, the range of the IFN-score gradually decreased with increasing prednisone dose, indicating that the suppression of IFN-score is dose-dependent. The decrease in IFN-score was most pronounced at the highest doses of 10 mg/day or more (Kruskal-Wallis, p=0.041, Mann-Whitney U 10 mg/day vs. 0mg/day, p=0.022).

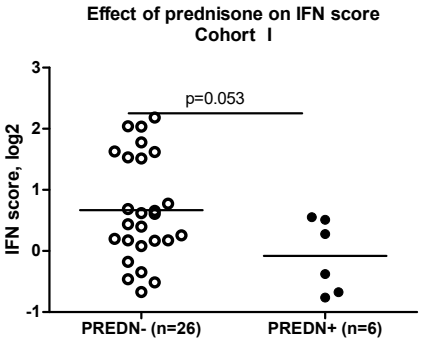


Figure 1 Effect of prednisone use on IFN-score in cohort I. In peripheral blood of 32 RA patients, gene expression levels of 8 interferon response genes were averaged to calculate the IFN-score. The IFN-score was evaluated in relation to prednisone use; Prednisone-treated patients ($PREDN^+$) exhibited a lower IFN-score than prednisone-untreated patients ($PREDN^-$).

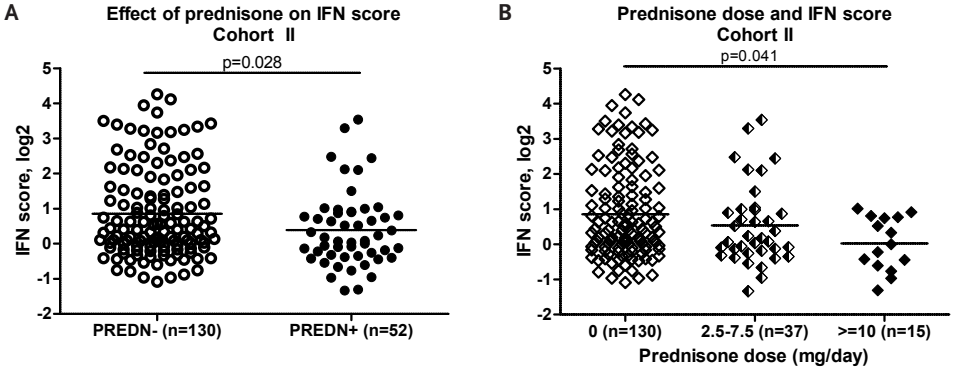


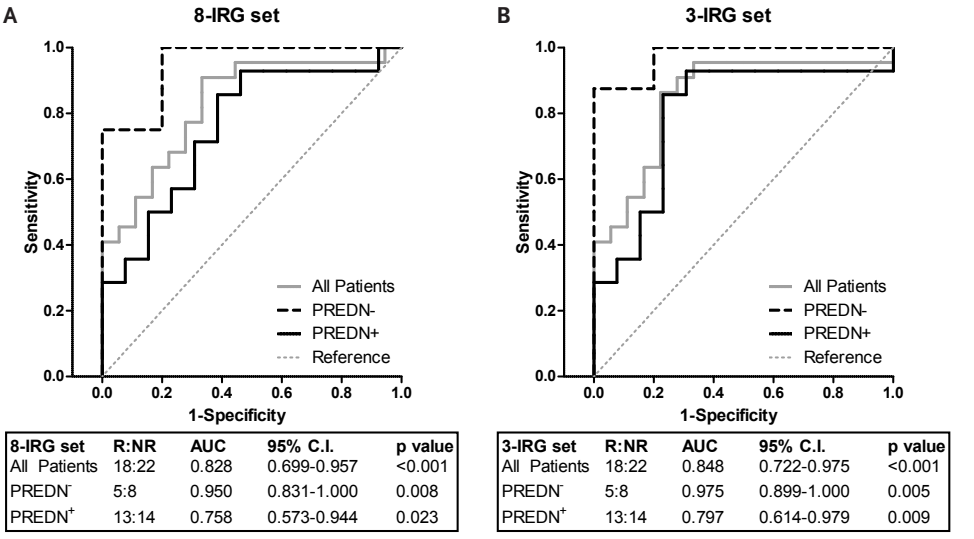
Figure 2 Effect of prednisone on the IFN-score validated in cohort II. In peripheral blood of 182 RA patients, gene expression levels of 8 interferon response genes were averaged to calculate the IFN-score. The IFN-score was evaluated in relation to prednisone use and prednisone dose. A) Comparison of IFN-score between prednisone-untreated ($PREDN^-$) and prednisone-treated ($PREDN^+$) RA patients; B) The relation between prednisone dose and IFN-score, assessed using Kruskal-Wallis.

Effect of prednisone treatment on the predictive value of the IFN-score in the outcome of rituximab treatment

The above results indicate that prednisone use is associated with a lower IFN-score in RA. We reasoned that the suppressive effect of prednisone on the IFN-score could have implications for the clinical utility of the IFN-score as predictor for the outcome of rituximab therapy in RA. Therefore, we studied the relation between prednisone use and the predictive value of the IFN-score in a cohort of 40 RA patients that were candidates for rituximab therapy (Cohort III).

The predictive value towards the clinical response to rituximab was determined for the 8-IRG-based IFN-score, as well as for the IFN-score based on 3 IRGs (EPSTI1, MX1 and RSAD2), which was previously described to give the most optimal performance as a predictor of rituximab response (3). As described before, no association of prednisone use itself as predictor for the outcome of rituximab treatment was found in this cohort (OR:2.0, 95% C.I.:0.49-8.20, P=0.335 (3)). As a measure of accuracy of the IFN-score in separating responders and non-responders, we performed Receiver Operating Characteristics (ROC) analysis on the whole group (n=40, 18 responders, 22 non-responders), the PREDN^{+} group (n=27, 13 responders, 14 non-responders) and PREDN^{-} group (n=13, 5 responders, 8 non-responders).

For the 8-IRG set, the group as a whole showed an area under the curve (AUC) of 0.828, which is considered very good. The group of PREDN^{+} patients alone revealed an AUC of 0.758 which is less than observed for the whole group. For the PREDN^{-} patients, the AUC reached an excellent value of 0.950 (**Figure 3A**).



8-IRG set	R:NR	AUC	95% C.I.	p value
All Patients	18:22	0.828	0.699-0.957	<0.001
PREDN^{-}	5:8	0.950	0.831-1.000	0.008
PREDN^{+}	13:14	0.758	0.573-0.944	0.023

3-IRG set	R:NR	AUC	95% C.I.	p value
All Patients	18:22	0.848	0.722-0.975	<0.001
PREDN^{-}	5:8	0.975	0.899-1.000	0.005
PREDN^{+}	13:14	0.797	0.614-0.979	0.009

Figure 3 ROC analyses of rituximab response prediction in in cohort III. The predictive value of the IFN-score in the outcome of rituximab treatment was assessed per patient subgroup based on prednisone treatment. A) ROC analysis on the 8-IRG set; B) ROC analysis on the highly predictive 3-IRG set.

The same ROC analyses for the optimally performing 3-IRG set revealed an AUC of 0.797 in the PREDN$^+$ group, again less than the AUC of 0.848 that was observed for the whole group. The PREDN$^-$ group reached an AUC of 0.975, which is equivalent to an excellent prediction (**Figure 3B**). At an IFN-score cutoff with a specificity of 100%, this corresponds to a sensitivity of 88% in PREDN$^-$, compared to a sensitivity of 41% in the whole group (**Figure 4A**). These findings indicate that stratification on prednisone use before measuring the expression of IRGs to predict the clinical outcome of rituximab treatment could dramatically improve the predictive power of the test.

A detailed analysis of the enhanced AUC in the PREDN$^-$ group revealed that the improved prediction of RTX response is a consequence of a larger difference in IFN-score between responders and non-responders in the PREDN$^-$ group, together with improvement of the IFN-score cutoff value. When using the IFN-score cutoff yielding 100% specificity in the whole group, the PREDN$^-$ group already displays an improved sensitivity of 63%, compared to the original 41% of the whole group (**Figure 4A**). When the IFN-score cutoff was selectively determined for the PREDN$^-$ group, sensitivity in this group was even further enhanced to 88% (**Figure 4A and 4B**). Altogether, stratification for prednisone use in this cohort resulted in correct classification of 100% of the responders and 50% of all non-responders (7/8 non-responders in the PREDN$^-$ group and 4/14 non-responders in the PREDN$^+$ group), compared to 100% of responders and 41% of the non-responders (9/22 non-responders) without stratification.

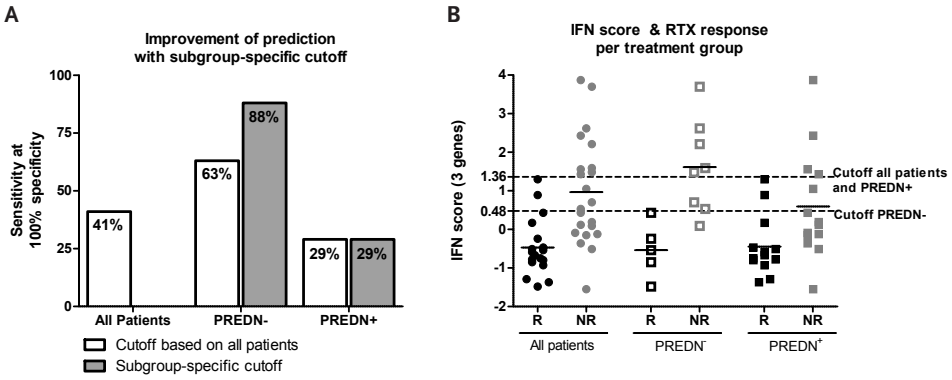


Figure 4 Subgroup-specific cutoffs for rituximab prediction. Detailed analysis of the improvement in rituximab response prediction upon stratification for prednisone use. A) Sensitivities of rituximab response prediction combined with 100% specificity, when using subgroup-specific cutoffs of the IFN-score, based on the ROC analyses per subgroup; B) IFN-scores per responder group and treatment-subgroup. Subgroup-specific cutoffs for 100% specificity are indicated with the dotted lines and are 1.36 for all patients and the PREDN$^+$ patients, and 0.48 for the PREDN$^-$ patients.

Discussion

Previous studies have shown that the IFN-score has clinical relevance by predicting the outcome of rituximab therapy; a high IFN-score reflecting increased IRG expression at baseline is associated with a poor clinical response to rituximab (2;3). In the present study, we demonstrated that the average IFN-score was consistently lower in prednisone-using patients compared to patients not using prednisone. As a consequence, rituximab response prediction based on the IFN-score was considerably improved when stratifying patients based on prednisone use. ROC analysis on the PREDN$^-$ group based on an IFN-score of 3 IRGs yielded an almost perfect AUC of 0.975, compared to 0.848 and 0.797 in all patients or prednisone users alone, respectively. This means that a test based on the 3-IRG IFN-score would correctly classify 98% of two PREDN$^-$ patients of two randomly drawn pairs, which is considered "excellent". Based on these data, non-response to rituximab could be predicted with a specificity of 100% and a sensitivity of 88% in PREDN$^-$ patients.

At the moment, the IFN-score-based rituximab prediction model seems to be the most discriminative test for rituximab response prediction and has already demonstrated clinical utility (3;11). The current data show that the model could be further optimized via stratification for prednisone use. GC therapy has proven to be a vital part in the management of RA (1) and is often prescribed as bridging therapy in between biologics to prevent or suppress inflammatory flares. The observation that the rituximab response prediction reached optimal predictive value in patients without current prednisone treatment suggests that the prednisone-related suppression of the IFN-score obscures the "genuine" intrinsic IRG expression, leading to the lower accuracy of prediction. Since elimination of prednisone use in RA patients would be practically intolerable, implementation of prednisone use and/or dose into the IFN-score-based prediction model might be an approach to optimize prediction of treatment outcome. Eventually it might be considered to taper or temporarily stop prednisone treatment to measure the "genuine" intrinsic IRG expression to predict the response to rituximab treatment, if the clinical condition of the patients allows that. The current data provide the first indication of the effect of medication history on response prediction. The study consisted of cross-sectional data and needs to be confirmed in a larger RA cohort that besides medication history also includes analysis on the influence of cumulative dosing and term of prednisone treatment.

Our findings on the in-vivo suppressive effect of prednisone on IRG expression in RA corroborates results from mechanistic studies that reported an effect of GCs on the type I IFN system. In systemic lupus erythematosus (SLE), methylprednisolone injection coincided with a decrease in plasmacytoid dendritic cells (pDCs), which are considered to be the main producers of IFNα in SLE (12;13). In RA, evidence is available for a role of both IFNα and IFNβ (14;15), indicating a broader cellular origin for these IFNs, making it unlikely that the prednisone-related IRG suppression in RA is caused solely by a decrease in pDCs.

Since GCs are able to interfere with the IRF3 and IRF9 pathways, thereby affecting IFNα/β induction and/or downstream IFN receptor (IFNAR) signaling, this could lead to suppression of both type I IFN production as well as downstream IRG induction. Such suppression is caused by the

interaction of GRIP1/NCOA2 –a cofactor of GC signaling– with IRF3 and IRF9, and subsequent interference between GR signaling and TLR signaling and IFNAR signaling, respectively (6;7). Additionally, it was demonstrated that GCs are able to induce expression of SOCS1 (16), a well-known inhibitor of JAK-STAT signaling, including type I IFN signaling (17). Because both TLR and JAK-STAT signaling are implicated in the regulation of type I IFN activity in RA (18;19), this may be an additional mechanism of the observed prednisone-related IRG suppression. However, our study was not aimed to unravel the mechanisms of GC-mediated type I IFN suppression, which is the objective of future studies.

Our observations raise questions regarding the relation between high baseline IRG expression and a poor response to rituximab. It is yet unclear whether high IRG expression is (in)directly causative for rituximab non-response or whether it is a related epiphenomenon. In case of a causative relation between high baseline IRG expression and rituximab non-response, it would be expected that prednisone use, as a suppressor of IRG expression, would lead to more responders. This was not observed in our cohort, as reflected by the described absence of a direct relation between prednisone use and the clinical response to rituximab (3). Moreover, we did not observe any bias in clinical parameters between the subgroups of prednisone use and RTX response. Since the numbers of patients per subgroup are rather small, this could be due to a lack of power. However, our data indicate that the difference in prediction accuracy between $PREDN^{-}$ and $PREDN^{+}$ patients is selectively due to prednisone-related IRG suppression in rituximab non-responders, resulting in false positive good responders in the $PREDN^{+}$ group, whereas responders from non-responders are almost perfectly distinguishable in the $PREDN^{-}$ group. Altogether, these observations indicate that IFN^{high} patients using prednisone might appear as IFN^{low} patients due to the prednisone-related IRG suppression, but still turn out to be non-responders to rituximab. This would in turn imply that the relation between high IRG expression and RTX non-responders is not a directly causative one.

Besides the association between baseline IRG expression and rituximab response, there are indications of pharmacodynamic differences during rituximab therapy as well. Vosslamber et al. provided evidence that RTX responders, i.e. patients with low baseline IRG expression, exhibited IRG upregulation after three months of therapy, whereas RTX non-responders did not (20), suggesting that type I IFN dynamics is related to the clinical outcome of rituximab treatment. It was hypothesized that high IRG expression before rituximab treatment could reflect an over-stimulated type I IFN system, incapable of further inducing the IRG expression that would be essential to reach a favorable response to rituximab. With regard to prednisone interference, one could speculate that this process of pathway saturation, possibly caused by extensive negative feedback or shortage of signaling proteins, could be synergistically enhanced by prednisone. This would then result in absence of IRG induction during rituximab therapy, despite relatively low IRG expression at baseline. Interestingly, the majority of patients in the study of Vosslamber et al. was using prednisone (82%), and patients were allowed to continue using it during rituximab therapy (20). Moreover, the observed pharmacological induction of IRG expression during rituximab therapy was described to be irrespective of clinical parameters such as prednisone use (20), suggesting it was persistent despite GC interference. This could in turn imply that the IRG expression as induced during rituximab treatment occurs via a different mechanism than the IRG expression at baseline, which has appeared sensitive to prednisone interference.

Our data might also be useful for other treatment regimens, as the relation between the IFN system and treatment response does not seem to be restricted to rituximab. E.g. for anti-TNF therapy with infliximab, dynamics of IRG expression appeared to be related to the clinical response, as non-responders showed an IRG upregulation during treatment whereas good responders did not (21;22). Furthermore, a genome-wide expression study revealed that high IRG expression before start of treatment with tocilizumab, an IL-6R blocker, is associated with a favorable response (23). The response patterns observed for these biologics are not in line with that for rituximab. Although the clinical relevance for these results need to be validated in independent studies, it may indicate that the status of the IFN system might have different clinical consequences in RA depending on the specific biologic that is used, i.e. the immune pathway that is modulated. Our findings on in-vivo interference of the IFN-system by prednisone may be equally relevant for the other biologic therapies and indications that are characterized by differential IFN activity. In these cases separate analysis of $PREDN^-$ and $PREDN^+$ patients could provide supportive value for these claims.

Conclusions

In conclusion, we have demonstrated that type I IFN activity in RA patients is suppressed upon prednisone use. Consequently, our findings reveal that the IFN-score-based model to predict the clinical outcome of rituximab can be optimized by implementation of prednisone use. This result provides an accurate system for response prediction of rituximab, thereby taking the paradigm of personalized medicine one step further.

Acknowledgements

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Chapter 2.3

A multi-parameter response prediction model for rituximab in rheumatoid arthritis

Tamarah D. de Jong*,
Jérémie Sellam*,
Rabia Agca, S
askia Vosslamber,
Birgit I. Witte,
Michel Tsang-a-Sjoe,
Johannes W. Bijlsma,
Alexandre E. Voskuyl,
Mike T. Nurmohamed,
Cornelis L. Verweij‡,
Xavier Mariette‡

* Both authors contributed equally
‡ Shared last authorship

Submitted for publication

Abstract

Objectives

To validate the IFN response gene (IRG) set for the prediction of non-response to rituximab in rheumatoid arthritis (RA) and assess the predictive performance upon combination of this gene set with clinical parameters.

Methods

In two independent cohorts of 93 (Cohort I) and 133 (Cohort II) rituximab-starting RA patients, baseline peripheral blood expression of eight selected IRGs was determined, and averaged into an IFN score. Individual predictive performance of the IFN score and clinical parameters was assessed by logistic regression. A multivariate prediction model was developed using a forward stepwise selection procedure. Patients with a decrease in disease activity score (ΔDAS28) ≥1.8 after 6 months of therapy were considered responders.

Results

A significantly higher IFN score was observed in RTX non-responders compared to RTX responders from both Cohort I and II, but this difference was most pronounced in patients who did not use prednisone (PREDN$^-$), as described before. Univariate analysis in Cohort I showed that baseline DAS28, IFN score and DMARD use were associated with non-response to rituximab, whereas positivity for IgM-RF and ACPA was associated with a good response to RTX. The multivariate model consisted of DAS28, IFN score and DMARD use, which showed an area under the curve (AUC) of 0.82. Validation of this model in Cohort II revealed a comparable AUC in PREDN$^-$negative patients (0.78), but AUC in PREDN$^-$positive patients was significantly lower (0.63), which seemed due to effect modification of the IFN score by prednisone.

Conclusions

The combination of predictive parameters provided a promising model for the prediction of non-response to rituximab, with possibilities for optimization via definition of the exact interfering effect of prednisone on IFN score.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease that may lead to permanent cartilage damage and bone destruction. The appearance of autoantibodies, such as rheumatoid factor (RF) and antibodies against citrullinated proteins (ACPA), sometimes years before disease onset, reflects the involvement of B cells already at the break of tolerance (1;2). Furthermore, accumulation of B cells, as well as of B cell-stimulating mediators, is observed in the RA synovium (3;4). Besides autoantibody production, B cells are thought to contribute to RA pathology by antigen presentation, T cell activation and cytokine production (3;5;6). The accumulated evidence of the role of B cells in RA has eventually led to the availability of B cell depletion therapy for RA patients.

Rituximab (RTX), a chimeric monoclonal antibody directed against the CD20 molecule on the surface of B cells, has been approved for use in RA patients who have failed or appeared intolerant to anti-TNF therapy (7). Binding of rituximab to CD20 results in effective depletion of naive, mature, and memory B cells, but not precursor B cells or plasma cells (8;9), supposedly by a combination of antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and apoptosis (10). Depending on the definition of response, approximately 30-40% of RA patients display a poor response to RTX therapy (9;11-13). Non-responding patients often receive RTX for at least 3 months, during which the disease progresses and joint damage continues. In order to prevent this, it is highly important to identify non-responders to RTX before start of therapy.

Many efforts have been made to find biomarkers for the response to rituximab. Clinical factors described to be associated with (non-)response to RTX are baseline disease activity, RF or ACPA positivity, high ACPA titers and a lower number of previous TNF inhibitors (14-19). Other predictive markers that have been described as predictors of RTX response involve the frequency of blood memory B cells, serum levels of the B cell-related chemokine CCL19 and the expression of interferon (IFN) response genes (IRG) (20-24). We have demonstrated that IRG expression at baseline was associated with a poor response to RTX (22-24). It has recently been shown that the performance of the IRG gene set could be further improved upon stratification on prednisone use (25).

Elevated IRG expression is a known feature in RA (26;27). Although its exact pathologic role in RA remains to be unraveled, presence of a signature was shown to be related to a poor response to rituximab (22;23) and putatively also to a good response to anti-TNF therapy and to tocilizumab (28;29).

In the study by Raterman et al., the IRG profile was found to outperform both seropositivity and the number of TNF inhibitors (22), suggesting that the IRG profile is a predictor independent of the other parameters. Consequently, combination of these parameters might further improve the prediction performance. In the current study, we aimed to validate the IRG biomarker in independent cohorts as well as to develop a response prediction model for RTX using both clinical parameters and IRG expression levels.

Methods

Patient recruitment

This study consisted of two independently collected cohorts. Cohort I consisted of 93 consecutive RA patients, who were recruited at the Amsterdam Center for Rheumatology and immunology, locations VUmc and Reade, Amsterdam, the Netherlands between 2006 and 2013. Thirty-nine of these 93 patients overlap with the study described by Raterman et al (22) ("group A"), whereas the remaining 54 patients were newly included ("group B"). We compared the clinical parameters between these two patient groups, which revealed no significant differences except RTX dose and swollen joint count (SJC28) (**Supplementary Table 1**). The effects of SJC28 on RTX non-response were similar for both groups (p value interaction term SJC28*group 0.21) and there were no differences in the parameters between the dose subgroups of group B and the complete group A. Therefore, we decided to merge both groups in order to gain power for the predictive analyses. Cohort II consisted of 133 patients from the SMART study (NCT01126541). This study is a 2-year, national, multicenter, randomized, open-label study conducted in France evaluating the efficacy and tolerability of two doses of RTX for re-treatment after one initial course of RTX, described previously (30). Details on each cohort are described in the supplementary methods (**Supplementary File 1**). From each patient, a PAXgene tube (PreAnalytix) was drawn and stored at -20°C until further use. This study was approved by the local ethics committees of the VU University medical center Amsterdam, Reade Amsterdam, the Netherlands, and by the the local ethics committee (Groupe Hospitalier Pitié-Salpêtrière) for SMART cohort use. All subjects gave their informed consent for the entire trial.

Treatment and clinical evaluation

Patients from Cohort I received either 500mg (n=13) or 1000 mg (n=80) RTX intravenously on days one and 15. The use of concomitant DMARDs, prednisolone or non-steroidal anti-inflammatory drugs (NSAIDs) was permitted. Patients from Cohort II received 1000mg RTX intravenously on days 1 and 15. Each patient in Cohort II received a stable dose of methotrexate (≥10 mg/week for at least 4 weeks). Steroids (prednisone or equivalent ≤10 mg/day) and NSAIDs were permitted if their doses had remained stable for at least 4 weeks and 2 weeks, respectively. All patients from both cohorts received methylprednisolone, acetaminophen and antihistamine as premedication.

After six months, presence of clinical response was defined as a decrease in disease activity score (ΔDAS28) ≥ 1.8. This cutoff was described to exceed random long-term fluctuation of DAS28 and therefore should reflect a more genuine therapeutic response than with the known response measures of ΔDAS ≥ 1.2 and the ΔDAS ≥ 0.6 which is used for EULAR moderate and good response (31). The overlap between responders and non-responders based on the ΔDAS28 cutoffs 1.8 and 1.2 and EULAR cutoffs are shown in **Supplementary table 2**.

RNA isolation, reverse transcription and pre-amplification of cDNA

RNA was isolated as described before (22). RNA (0.5 µg) was reverse transcribed into cDNA using a RevertAid Hminus cDNA synthesis kit (ThermoFisher Scientific) according to the manufacturer's instructions. A single aliquot of each cDNA sample, equivalent to 12.5 ng RNA, was first subjected to 14 cycles of Specific Target Amplification using a 0.2X mixture of all Taqman Gene

Expression assays in combination with the Taqman PreAmp Master Mix (Applied Biosystems). Following pre-amplification, the samples were diluted 1:5 (v/v) in Tris-EDTA buffer, pH 8.0.

Fluidigm arrays

Custom-designed TaqMan®assays for each gene were supplied by Applied Biosystems. qPCR analysis was performed at ServiceXS (ServiceXS B.V., Leiden, The Netherlands) using the 96.96 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation), according to the manufacturer's instructions. Thermal cycling and real-time imaging of the BioMark array was performed on the BioMark instrument, and C_T values were extracted using the BioMark Real-Time PCR analysis software. Relative quantities were calculated using the standard curve method, using GAPDH as a housekeeping gene. Expression levels were ^{2}log-transformed.

Type I IFN response gene expression

Gene expression was determined of 8 type I IFN response genes (IRGs), previously described to be predictive for the non-response to RTX (22). As described before, the expression levels of all 8 IRGs were averaged, which will be referred to as the IFN score. After careful technical control, previously described microarray and qPCR measurements (22) were combined with the multiplex qPCR described above by median centering.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 22. Variables with more than 5% missing data were not included. Comparison of cohorts was performed using either Mann-Withney or Chi-square (χ^2), where appropriate. Comparison of IFN scores between responders and non-responders was performed using an independent samples t-test. Variables were assessed for their ability to predict the non-response to RTX by performing logistic regression with ΔDAS<1.8 as outcome. Variables significantly associated with the non-response to RTX in the univariate logistic regression analysis were included in the multivariate analysis, which was performed using forward stepwise logistic regression (p-value entry 0.05, p-value removal 0.10). Nagelkerke's R^2 was used to determine the proportion of variation explained by the model. Discriminative performance was assessed using Received Operating Characteristics (ROC) analysis. The Area Under the Curve (AUC) of different ROC curves were compared using the roccomp syntax in Stata version 12. P values below 0.05 were considered significant.

Results

Patient characteristics

Patient characteristics are shown in **Table 1**. Non-response (ΔDAS28 <1.8) after six months of RTX treatment was 65% in Cohort I and 51% in Cohort II (p=0.046). In general, patients from Cohort II appeared to exhibit more active disease, indicated by a higher baseline DAS28, a higher tender joint count and a higher incidence of erosions compared to Cohort I (p<0.001 for all three variables). On the other hand, patients from Cohort I were more often IgM-RF positive and/or ACPA positive (IgM-RF, p=0.009; ACPA p=0.081; positivity for both, p=0.015). Medication status was different in MTX use (58% in Cohort I, 100% in Cohort II, p<0.001) and prednisone use (65% in Cohort I and 77% in Cohort II, p=0.045).

Table 1 Baseline patient characteristics of the Cohort I and Cohort II

	Cohort I (n=93)	Cohort II (n=133)	I vs. II MW or χ^2, p value
Demographics			
Female, n (%)	78 (84)	110 (83)	0.82
Age, mean ± SD	57.0 ± 12.3	55.0 ± 11.6	0.31
Disease characteristics			
Disease duration, mean ± SD	11.5 ± 9.2	13.2 ± 9.3	0.11
DAS28, mean ± SD	5.6 ± 1.1	6.2 ± 0.9	<0.001
VAS, mean ± SD	65.0 ± 18.3	65.2 ± 17.4	0.99
TJC28, mean ± SD	10.0 ± 6.6	14.9 ± 6.7	<0.001
SJC28, mean ± SD	9.6 ± 5.2	10.7 ± 4.3	0.11
Erosions, n (%)	61 (68)†	118 (89)	<0.001
Laboratory parameters			
CRP, median (range)	10.0 (1.0-121.0)	11.0 (30.0-139.0)	0.63
CRP > 10, n (%)	45 (49)‡	69 (52)	0.66
ESR, median (range)	27.0 (2.0-122.0)	26.0 (2.0-102.0)	0.65
ESR > 20, n (%)	55 (59)	86 (65)	0.40
IgM-RF and ACPA negative, n (%)	9 (10)	26 (20)	0.04
IgM-RF positive, n (%)	75 (81)	86 (65)	0.009
ACPA positive, n (%)	80 (86)	102 (77)	0.08
IgM-RF and ACPA positive, n (%)	71 (76)	81 (61)	0.02
IFNscore, mean ± SD	0.37 ± 1.35	0.17 ± 1.19	0.41
Medication			
Number of previous biologicals, mean ± SD	1.8 ± 1.0	0.95 ± 0.21	
DMARD use, n (%)	55 (62)§	133 (100)	<0.001
Prednisone use, n (%)	58 (65)§	103 (77)	0.05
Prednison dose, median (range)	10.0 (5.0-30.0)	NA	NA
Prednisone dose > 5mg/day, n (%)	44 (49)	69 (55)¶	0.44
MTX use, n (%)	52 (58)§	133 (100)	<0.001
MTX dose, median (range)	25.0 (2.5-30.0)	15.0 (8.0-25.0)	<0.001
SSZ use, n (%)	10 (11)§	0 (0)	NA
HCQ use, n (%)	9 (10)§	0 (0)	NA
RTX dose 1000mg, n (%)*	80 (86)	133 (100)	<0.001
Rituximab response			
ΔDAS28, mean ± SD	-1.3 ± 1.3	-1.7 ± 1.3	0.03
ΔDAS >1.8, n (%)	33 (36)	65 (49)	0.05
EULAR non-response, n (%)	30 (32)	35 (26)	0.38
EULAR moderate response, n (%)	48 (52)	81 (61)	
EULAR good response, n (%)	15 (16)	17 (13)	

** RTX dose was either 500mg or 1000mg; †data missing from 3 patients; ‡data missing from 1 patient; § data missing from 4 patients; ¶ data missing from 7 patients. DAS28, disease activity score of 28 joints; VAS, Visual Analogue Scale; TJC28, Tender Joint Count of 28 joints; SJC28, Swollen Joint Coint of 28 joints; CRP, C Reactive Protein; ESR, Erythrocyte Sedimentation Rate; IgM RF, IgM Rheumatoid Factor; ACPA, anti-cyclic citrullinated protein; IFN, interferon; DMARD, disease-modifying anti-rheumatic drugs; MTX, methotrexate; SSZ, sulfasalazine; HCQ, hydroxychloroquine; NA, not available; ND, not determined; MW, Mann Whitney U test.*

Validation of the IFN response gene biomarker

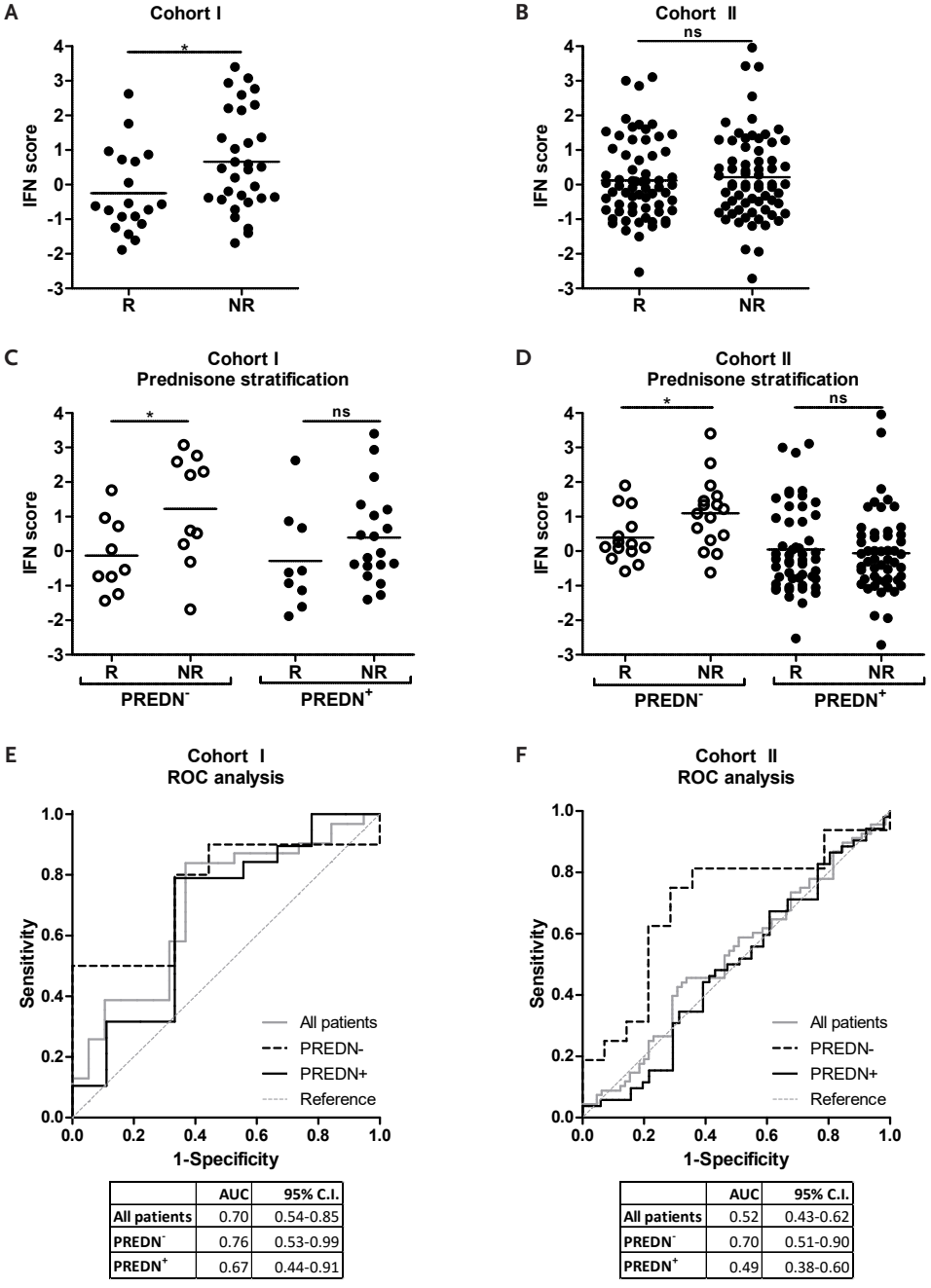
First, we validated the earlier described relation between IFN response gene (IRG) expression and poor response to RTX (22) in the newly included patient subgroup from Cohort I (n=54, IRG expression data available for 50 patients) and the patients from Cohort II (n=133).

As displayed in **Figure 1A** and **1B**, we observed a significantly higher IFN score in non-responders compared to responders in the Cohort I subgroup (p=0.026), but not in Cohort II (p=0.65). However, stratification on prednisone use revealed that the relation between IFN score and non-response was more pronounced in patients not using prednisone ($PREDN^-$) patients compared to prednisone-using patients ($PREDN^+$), as described before (25) (**Figure 1C** and **1D**). Concordantly, ROC analyses displayed higher AUCs in the $PREDN^-$ selection (Cohort I 0.76 and Cohort II 0.70) than in the $PREDN^+$ selection (0.67 and 0.49, respectively) or in the complete cohort (0.70 and 0.52, respectively) (**Figure 1E** and **1F**). Interestingly, prednisone use was not a significant effect modifier or confounder of IFN score neither in the subgroup of Cohort I, nor in the complete Cohort I, but it did appear a significant effect modifier in Cohort II (p value interaction term p=0.047). In line with this effect modification, the difference in AUC between $PREDN^-$ and $PREDN^+$ approached significance for Cohort II (p=0.072), but not for Cohort I (p=0.63).

Altogether, these data validate the IFN score as a predictor of non-response to RTX in $PREDN^-$ patients, but also emphasize the earlier described observation that prednisone use interferes with this association (25).

Univariate analysis

In order to identify additional baseline parameters that predict the non-response to RTX, we performed univariate logistic regression using the complete Cohort I (n=93) and Cohort II (n=133). As shown in **Table 2**, predictors associated with non-response to RTX in Cohort I were DMARD use, low baseline DAS28 and a high IFN score. On the other hand, positivity for IgM-RF and ACPA was associated with a good response to RTX. DAS28 and positivity for IgM-RF and ACPA were also found associated with non-response in Cohort II, with similar odds ratios (ORs) as for Cohort I (see **Table 2**). Since prednisone was an effect modifier in the association between IFN score and RTX non-response in Cohort II, we also separately analyzed the $PREDN^-$ and $PREDN^+$ subgroups of this cohort. As expected, IFN score appeared only significantly associated with non-response with the $PREDN^-$ subgroup of Cohort II, but not in the $PREDN^+$ subgroup. Conversely, DAS28 and positivity for IgM-RF and ACPA were significant predictors in the $PREDN^+$ subgroup of Cohort II, but not in the $PREDN^-$ subgroup, which might be due to a lack of power.



	AUC	95% C.I.
All patients	0.70	0.54-0.85
PREDN⁻	0.76	0.53-0.99
PREDN⁺	0.67	0.44-0.91

	AUC	95% C.I.
All patients	0.52	0.43-0.62
PREDN⁻	0.70	0.51-0.90
PREDN⁺	0.49	0.38-0.60

Figure 1 Validation of the previously described relation between IFN response gene (IRG) expression and RTX response. A) and B) IFN scores from RTX responders and non-responders from Cohort I (A) and Cohort II (B). C) and D) IFN scores in patients from Cohort I (C) and Cohort II (D) with stratification on prednisone use. E) ROC analyses of the IFN score in all 50 patients of Cohort I, as well as selectively in the PREDN⁻ and PREDN⁺ groups. Information on prednisone use was missing for 3 patients. F) ROC analyses of the IFN score in the complete group of Cohort II, as well as selectively in the PREDN⁻ and PREDN⁺ groups. * p<0.05; ns, nonsignificant; AUC, Area Under the Curve; C.I. Confidence Interval.

Table 2 Univariate analysis of RTX non-response prediction in Cohort I. Odds ratios (OR) are only displayed for p values≤0.05. r^2 indicates Nagelkerke r^2.

	Cohort I (n=93, 33 R, 60 NR)			Cohort II, all patients (n=133, 65 R, 68 NR)			Cohort II, PREDN$^-$ (n=30, 14 R, 16 NR)			Cohort II, PREDN$^+$ (n=103, 51R, 52 NR)		
	OR (95% C.I.)	P value	R^2	OR (95% C.I.)	P value	R^2	OR (95% C.I.)	P value	R^2	OR (95% C.I.)	P value	R^2
Demographics												
Female sex		0.44			0.73			0.92			0.77	
Age in years		0.66			0.088			0.57			0.096	
Disease characteristics												
Disease duration in years		0.66			0.48							
DAS28	0.50 (0.30-0.79)	0.003	0.14	0.41 (0.26-0.64)	<0.001	0.18		0.11		0.38 (0.22-0.64)	<0.001	0.20
Presence of erosions		0.80			0.86			NA			0.80	
Laboratory parameters												
CRP*		0.47			0.28			0.53			0.94	
CRP >10		0.85			0.66			0.29			0.93	
ESR*		0.29		0.61 (0.39-0.96)	0.033	0.048		0.54		0.59 (0.36-0.98)	0.041	0.057
ESR >20		0.51			0.15			0.39			0.24	
IgM-RF and ACPA negative		0.10			0.11			0.92			0.075	
IgM-RF and ACPA positive	0.22 (0.06-0.80)	0.02	0.096	0.38 (0.19-0.79)	0.009	0.069		0.55		0.33 (0.15-0.75)	0.008	0.093
IFN score	1.8 (1.2-2.8)	0.005	0.15		0.65		2.7 (1.0-7.3)	0.052	0.18		0.66	
Medication												
Number of previous biologics		0.31			NA			NA			NA	
DMARD use	4.5 (1.8-11.5)	0.001	0.16		NA			NA			NA	
Prednisone use		0.93			0.78			NA			NA	
Prednisone dose		0.78			NA			NA			NA	
Prednisone dose >5mg/day					0.55			NA			0.51	
RTX dose 1000mg		0.70			NA			NA			NA	

** variables were assessed as log-transformed (natural log). R, responder; NR, non-responder.*

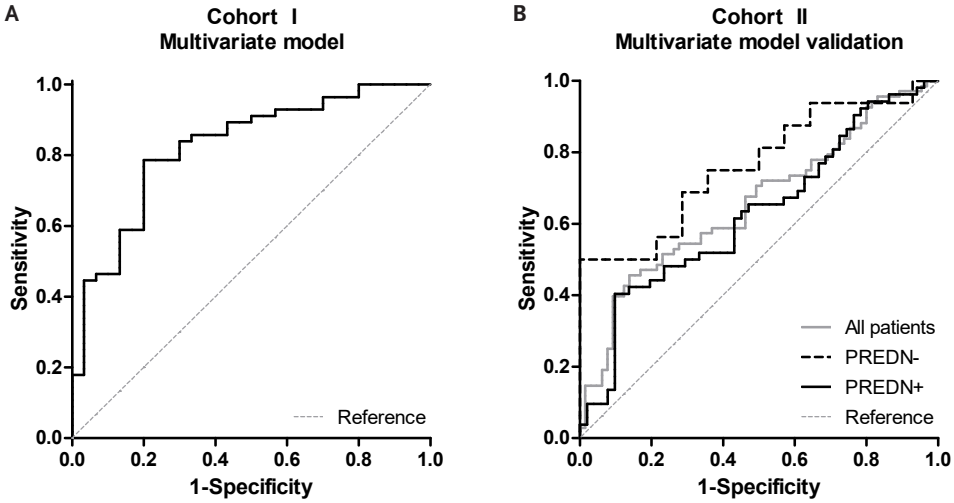
Multivariate analyses and validation in an independent cohort

A multi-parameter prediction model for the non-response to RTX was developed by a forward stepwise selection procedure using the significant predictors from the univariate analyses on Cohort I. Using an inclusion p value of 0.05 and a removal p value of 0.10, the final model consisted of baseline DAS28, IFN score and DMARD use (**Table 3**). This yielded a Nagelkerke R^2 of 0.38, which was considerably higher than for each variable individually (**Table 2**). As shown in **Figure 2A**, ROC analysis revealed a good AUC of 0.82 (95% C.I. 0.73-0.92). When aiming for maximum specificity with a sensitivity of at least 30%, the model displayed 97% specificity (correct classification of 30/31 responders) and 45% sensitivity (correct classification of 26/58 non-responders), at a cutoff of 0.85.

In order to validate the multivariate prediction model, we applied the logistic regression formula acquired with Cohort I to Cohort II. Of note, DMARD use was always positive in this cohort. This analysis resulted in an ROC curve with an AUC of 0.66 (95% C.I. 0.57-0.75) (**Figure 2B**), which was significantly lower than the AUC found for Cohort I (p=0.013). Separate analysis of this model in the $PREDN^-$ and $PREDN^+$ subgroups of Cohort II revealed an AUC of 0.76 (95% C.I. 0.59-0.94) for the $PREDN^-$ group and 0.63 (95% C.I. 0.52-0.74) for the $PREDN^+$ group (**Figure 2B**). Using the optimal cutoff of 0.85, as determined in Cohort I, the $PREDN^-$ group displayed 100% specificity and 50% sensitivity, which is similar to Cohort I, but the $PREDN^+$ subgroup reached only 90% specificity with 17% sensitivity.

Table 3 Multivariate analysis of RTX non-response prediction in cohort I and cohort II

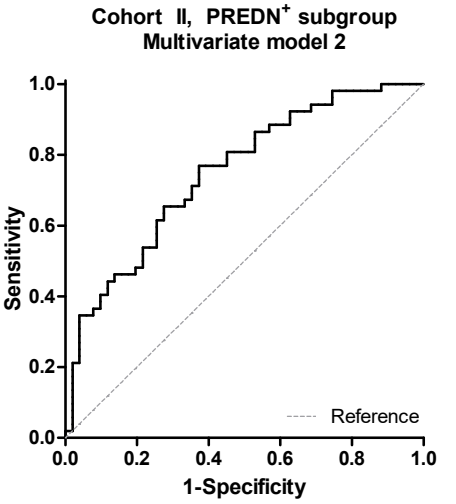
	Regression coefficient β	OR (95% C.I.)	P value
Cohort I			
DAS28	-0.95	0.39 (0.21-0.73)	0.004
IFN score	0.81	2.2 (1.3-3.7)	0.002
DMARD use	0.94	2.5 (0.9-7.5)	0.091
Cohort II, all patients			
DAS28	-0.90	0.41 (0.26-0.64)	<0.001
IFN score	0.074	1.08 (0.80-1.46)	0.632
Cohort II, $PREDN^-$			
DAS28	-0.78	0.46 (0.18-1.16)	0.101
IFN score	1.06	2.9 (1.0-8.3)	0.049
Cohort II, $PREDN^+$			
DAS28	-0.98	0.38 (0.22-0.64)	<0.001
IFN score	-0.083	0.92 (0.65-1.30)	0.635



R:NR	AUC	95% C.I.
30:56	0.82	0.73-0.92

	R:NR	AUC	95% C.I.
All Patients	65:68	0.66	0.57-0.75
PREDN$^-$	14:16	0.78	0.59-0.94
PREDN$^+$	51:52	0.63	0.52-0.74

Figure 2 ROC curves of the multivariate prediction model containing 3 parameters including the IFN score. A) Data from Cohort I. Final analysis was performed on 89 patients. B) Data from the complete Cohort II and with stratification on prednisone use.



R:NR	AUC	95% C.I.
51:52	0.75	0.66-0.84

Figure 3 ROC curve of multivariate prediction model 2 developed in the PREDN$^+$ subgroup of Cohort II.

Development of an alternative model for PREDN$^+$ patients in Cohort II

As the performance of the multivariate model in the PREDN$^+$ subgroup of Cohort II was significantly lower compared to Cohort I (p=0.007), we decided to develop an alternative model specifically using this subgroup. The forward stepwise selection procedure using the significant predictors from the univariate analysis (see **Table 2**) resulted in a multivariate model consisting of DAS28 and positivity for RF and ACPA, which showed a Nagelkerke R^2 of 0.25. ROC analysis revealed an AUC of 0.75 for this model, which was considerably better than the 0.66 of the previous model in this group (**Figure 3**). The performance of this particular model was lower than the first model in all other groups, although it also appeared better in the PREDN$^+$ subgroup compared to the PREDN$^-$ subgroup of Cohort I (**Supplementary Table 3**). Altogether, it appears that the optimal combination of predictive parameters might depend on prednisone status.

Discussion

Various predictors for the (non-)response to rituximab have been described independently (14-24). In the present study, we used two independent cohorts in which we confirmed some of those parameters and demonstrated that combining them greatly improves the predictive performance.

Considering the high heterogeneity of RA, it is to be expected that non-response to therapy is not determined by one single variable, but rather by a combination of variables. Since the cohort I consisted of 33 responders, the maximum number of variables in the model was restricted to three. However, using only DAS28, IFN score and DMARD use already revealed a good AUC of 0.82. Analysis selectively on the PREDN$^+$ subgroup of cohort II displayed considerable overlap with the analyses on cohort I, but did not include the IFN score, and the final multivariate model consisted of DAS28 and positivity for IgM-RF and ACPA, which yielded an AUC of 0.76. This indicates that the optimal combination of variables, particularly inclusion of IFN score in the model, might depend on prednisone status. A meta-analysis on a larger cohort might allow for combining more than three variables and could increase the predictive performance even further.

Since we defined ΔDAS28>1.8 as the outcome for good response, it is not entirely surprising that baseline DAS28 was found as a predictor for the outcome. Patients with a high DAS28 at baseline will reach a change of 1.8 more easily than patients who start therapy at a relatively low DAS28, which is reflected in the negative association of DAS28 with non-response.

The observed positive relations of RF and ACPA positivity with RTX response confirm previously described findings (14;17-19). Autoantibody positivity in general might be a reflection of B cell activity and consequently indicate high sensitivity to B cell depletion by RTX, which corroborates with the positive relation of RF and/or ACPA positivity with RTX response. Although the major producers of antibodies, the plasma cells, do not express CD20 and therefore are supposedly insensitive to RTX (8), multiple studies have reported a decrease in both RF and ACPA after RTX treatment (14;32-34). This suggests that these autoantibody-secreting plasma cells could be, at least partly, short-lived plasma cells and might be affected by the depletion of the other B cell subsets.

DMARD use at baseline, which mainly consisted of MTX use, appeared associated with RTX non-response. Hypothetically, pre-treatment with a DMARD could decrease the DAS28 at baseline, which in turn could decrease the chance to reach a ΔDAS28>1.8 response. Since the majority of patients in cohort I and all patients from our cohort II received MTX treatment, we could not study this in further detail.

Even though we observed interference of prednisone use on the IFN score as described before (25), the IFN score remained highly significant in both the univariate analyses and the multivariate analyses of Cohort I. This also confirms the previously described finding that the IFN score would outperform other predictive variables (22). The mechanism by which a high IFN score could be associated with a poor response to rituximab still remains to be elucidated, but the multivariate analyses suggest that it occurs independently of autoantibody positivity and disease activity. It has been suggested that the RA pathology in patients with a high IFN score could be less B-cell dependent compared to patients with a low IFN score (35). Alternatively, high IFN activity might affect B cell differentiation, leading to higher numbers of rituximab-insensitive plasmablasts (35;36).

Another interesting hypothesis can be suggested based on recent work from Mauri and colleagues, who have demonstrated cross-talk between pDC and regulatory B cells (Bregs) (37). It was shown that a low dose of IFN was necessary for Breg activation, while high IFN activity impaired Breg function. Although Bregs are also depleted by RTX, it is possible that a clinical response can be achieved in IFN^{low} patients because of rapid Breg restoration (37), whereas Breg function could remain impaired in IFN^{high} patients, hence leading to a poor response.

The difference in predictive performance of IFN score between Cohort I and Cohort II suggests that cumulative prednisone dosing could increase the suppressive effect of prednisone on IFN score. As part of the inclusion criteria, the prednisone-using patients from Cohort II had been on a stable prednisone dose for at least 4 months, whereas this was not restricted for the patients from Cohort I. Although chronic prednisone use is common in RA, information on the term of prednisone treatment and dose stability was not available for Cohort I, hence it is likely that there are patients included who just started prednisone treatment or recently tapered the dose. This could explain the observation that prednisone was a significant effect modifier of the relation between IFN score and RTX non-response in cohort II, but not in cohort I, as also reflected by the difference in AUCs for IFN in both cohorts. Future studies should focus on the effect of actual and cumulative dosing of prednisone on the IFN signature, in order to strictly define the window within which the IFN score could be successfully utilized as a predictor for RTX non-response.

In conclusion, using two relatively large independent cohorts, we demonstrated that the combination of predictive parameters provides a promising model for the prediction of RTX non-response, with possibilities for optimization via definition of the exact interfering effect of prednisone use and dose on IFN score. Altogether, this study provided promising data for potential personalized treatment of RA.

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Supplementary material

Supplementary methods

Cohort I consisted of 93 consecutive RA patients, according to the 1988 revised American College of Rheumatology (ACR) criteria for the diagnosis of RA (**38**) who were recruited at the Amsterdam Center for Rheumatology and immunology, locations VUmc and Reade, Amsterdam, the Netherlands between 2006 and 2013. All patients were candidates for RTX therapy because of their moderate or high disease activity (Disease Activity Score for 28 joints (DAS28) >3.2) despite disease-modifying anti-rheumatic drugs (DMARD) treatment and previous therapy with Tumor Necrosis Factor (TNF) inhibitors. At the moment of blood collection, patients were off TNF inhibitor therapy for at least four weeks and had not received their first RTX dose yet.

Cohort II consisted of 133 patients from the SMART study (NCT01126541). This study is a 2-year, national, multicenter, randomized, open-label study evaluating the efficacy and tolerability of two doses of RTX for re-treatment after one initial course of RTX at a usual dose (1000 mg on days 1 and 15) described previously (30). A total of 224 patients with RA for at least 6 months and fulfilling the ACR 1987 criteria were included. All patients had active disease, defined by a Disease Activity Score in 28 joints using the C reactive protein (CRP) (DAS28-CRP) > 3.2 and ≥ 6/66 swollen and ≥ 6/68 tender joints, or a CRP≥10 mg/L, or an erythrocyte sedimentation rate (ESR) ≥ 28 mm/h. Each patient had experienced an inadequate response or intolerance to TNF inhibitors; or had contraindications to TNF inhibitors. Patients had discontinued TNF inhibitor therapy for at least 4 weeks. In this ancillary study, 133 patients from the 224 were included due to the availability of the blood samples.

Supplementary Tables

Supplementary Table 1 Clinical characteristics of the two patients groups constituting Cohort I.

	A (n=39)	B (n=54)	Combined (n=93)	A vs. B, MW or χ^2, p value
Demographics				
Female, n (%)	33 (85)	45 (83)	78 (84)	0.87
Age, mean ± SD	57.1 ± 11.1	57.0 ± 13.2	57.0 ± 12.3	0.76
Disease characteristics				
Disease duration, mean ± SD	10.7 ± 9.7	12.1 ± 8.8	11.5 ± 9.2	0.23
DAS28, mean ± SD	5.8 ± 1.1	5.4 ± 1.0	5.6 ± 1.1	0.096
VAS, mean ± SD	66.2 ± 19.8	64.0 ± 17.2	65.0 ± 18.3	0.30
TJC28, mean ± SD	11.5 ± 7.0	8.9 ± 6.1	10.0 ± 6.6	0.099
SJC28, mean ± SD	12.2 ± 4.00	7.7 ± 5.2	9.6 ± 5.2	<0.001
Erosions, n (%)	27 (69)	34 (67)†	61 (68)†	0.77
Laboratory parameters				
CRP, median (range)	12.0 (1.0-66.0)	10.0 (1.0-121.0)	10.0 (1.0-121.0)	0.49
CRP > 10, n (%)	21 (54)	24 (45)‡	45 (49)‡	0.42
ESR, median (range)	24.0 (3.0-119.0)	27.5 (2.0-122.0)	27.0 (2.0-122.0)	0.34
ESR > 20, n (%)	22 (56)	33 (61)	55 (59)	0.65
IgM-RF and ACPA negative, n (%)	3 (8)	6 (11)	9 (10)	0.58
IgM RF positive, n (%)	30 (77)	45 (83)	75 (81)	0.44
ACPA positive, n (%)	36 (92)	44 (82)	80 (86)	0.14
IgM-RF and ACPA positive, n (%)	30 (77)	41 (76)	71 (76)	0.91
IFNscore, mean ± SD	0.43 ± 1.28	0.32 ± 1.41	0.37 ± 1.35	0.47
Medication				
Number of previous biologicals, mean ± SD	1.6 ± 1.0	1.8 ± 1.0	1.8 ± 1.0	0.41
DMARD use, n (%)	26 (67)	39 (58)§	55 (62)§	0.40
Prednisone use, n (%)	28 (72)	30 (60)§	58 (65)§	0.25
Prednison dose, median (range)	10.0 (5.0-30.0)	8.8 (5.0-20.0)	10.0 (5.0-30.0)	0.76
MTX use, n (%)	25 (64)	27 (54)§	52 (58)§	0.34
MTX dose, median (range)	20.0 (2.5-30.0)	25.0 (5.0-30.0)	25.0 (2.5-30.0)	0.41
SSZ use, n (%)	6 (15)	4 (8)§	10 (11)§	0.27
HCQ use, n (%)	4 (10)	5 (10)§	9 (10)§	0.97
RTX dose 1000mg, n (%)*	39 (100)	41 (76)	80 (86)	0.001
Rituximab response				
ΔDAS28, mean ± SD	-1.3 ± 1.2	-1.3 ± 1.3	-1.3 ± 1.3	0.69
ΔDAS >1.8, n (%)	12 (31)	21 (39)	33 (36)	0.42
EULAR non-response, n (%)	16 (41)	14 (26)	30 (32)	0.30
EULAR moderate response, n (%)	18 (46)	30 (56)	48 (52)	
EULAR good response, n (%)	5 (13)	10 (19)	15 (16)	

** RTX dose was either 500mg or 1000mg; †data missing from 3 patients; ‡data missing from 1 patient; § data missing from 4 patients. DAS28, disease activity score of 28 joints; VAS, Visual Analogue Scale; TJC28, Tender Joint Count of 28 joints; SJC28, Swollen Joint Coint of 28 joints; CRP, C Reactive Protein; ESR, Erythrocyte Sedimentation Rate; IgM-RF, IgM Rheumatoid Factor; ACPA, anti-cyclic citrullinated protein; IFN, interferon; MTX, methotrexate; MW, Mann-Whitney U test; SSZ, sulfasalazine; HCQ, hydroxychloroquine.*

Supplementary Table 2 Numbers of patients with response and non-response to rituximab, according to the ΔDAS28 ≥1.8 cutoff, and the overlap with ΔDAS28 ≥1.2 response and EULAR response in both cohorts.

Cohort I					
ΔDAS28 response			**EULAR response**		
			Good	**Moderate**	**Poor**
ΔDAS28 ≥1.8		33	13	20	0
ΔDAS28 <1.8	ΔDAS28 ≥1.2	17	2	15	0
	ΔDAS28 <1.2	43	0	13	30

Cohort II					
ΔDAS28 response			**EULAR response**		
			Good	**Moderate**	**Poor**
ΔDAS28 ≥1.8		65	17	48	0
ΔDAS28 <1.8	ΔDAS28 ≥1.2	20	0	20	0
	ΔDAS28 <1.2	48	0	13	35

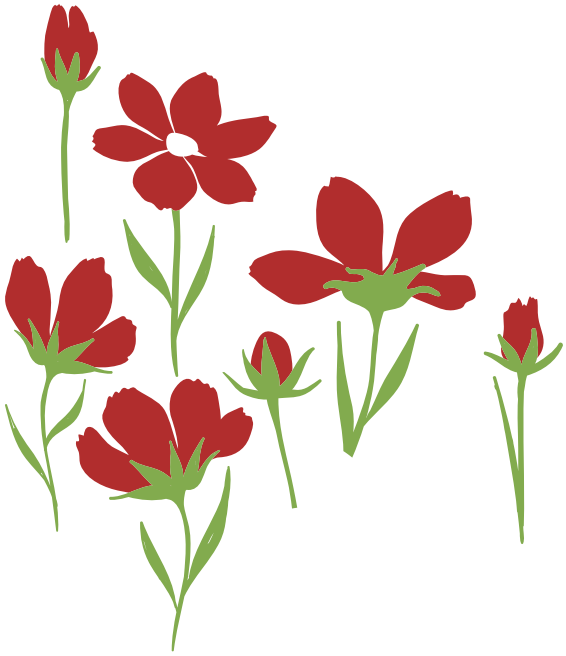
Supplementary Table 3 performance of the additional multivariate prediction model developed using the PREDN$^+$ selection of Cohort II in the other subgroups and Cohort I.

		AUC (95% C.I.)	
		Model 1*	**Model 2***
Cohort I	All patients	0.82 (0.73-0.92)	0.73 (0.63-0.84)
	PREDN$^-$	0.81 (0.63-0.98)	0.63 (0.42-0.84)
	PREDN$^+$	0.82 (0.71-0.93)	0.77 (0.65-0.90)
Cohort II	All patients	0.66 (0.57-0.75)	0.74 (0.66-0.82)
	PREDN$^-$	0.76 (0.59-0.94)	0.70 (0.51-0.89)
	PREDN$^+$	0.63 (0.52-0.74)	0.75 (0.66-0.84)

** Model 1 contains the variables DAS28, IFN score and DMARD use and model 2 contains the variables DAS28 and positivity for IgM-RF and ACPA.*

Chapter 3

Molecular characterization of the type I interferon signature in rheumatic diseases



Chapter 3.1

Differential mechanism of type I interferon response induction by serum from rheumatoid arthritis and systemic lupus erythematosus patients

Tamarah D. de Jong,
Saskia Vosslamber,
Maija-Leena Eloranta,
Lars Rönnblom,
Hetty J. Bontkes,
E. Mantel,
Irene E. Bultink,
Alexandre E. Voskuyl,
Cornelis L. Verweij

Manuscript in preparation

Abstract

Background

A role for type I interferon (IFN) is suggested in the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Whereas the mechanism of induction of IFN activity in SLE is largely elucidated, this remains to be determined for RA. This study aims to investigate the presence of an IFN inducing factor in RA serum.

Methods

Peripheral blood mononuclear cells (PBMCs) from healthy individuals were exposed to serum from RA (n=18) or SLE (n=25) patients. Expression of three IFN response genes (IRGs) was determined by qPCR after 4h and 8h incubation with 25% patient serum. To study the involvement of new protein synthesis, part of the samples was co-cultured with cycloheximide. Moreover, IFNα protein production was measured by immunoassay after 20h incubation with 5% patient serum. Samples were also co-cultured with apoptotic or necrotic cell material, as this has proven to enhance IFNα production by SLE serum. All cultures were performed with healthy donor serum (NHS) as a negative control.

Results

Both RA and SLE sera displayed interferogenic properties. SLE serum displayed IRG induction after 4h incubation, which remained high after 8h and was not inhibited by cycloheximide treatment, indicating that it occurs independently of *de novo* protein synthesis. RA serum induced IRG induction only after 8h, which was inhibited upon cycloheximide treatment, suggesting an indirect induction process. As expected, the IRG induction by SLE serum was found to be related to the induction of IFNα protein production –which was increased in SLE serum versus NHS– as well as to positivity for antinuclear antibodies. Only a small proportion of the RA serum samples marginally induced IFNα protein production.

Conclusion

Altogether, these results indicate that IRG induction in RA occurs via another mechanism than in SLE.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the joint which may lead to cartilage and bone destruction. RA manifests itself as a heterogeneous disease with a clinical spectrum ranging from mild to severe disease. The heterogeneity most likely has its origin in the multifactorial nature of the disease, whereby specific combinations of genetic risk factors together with an appropriate environmental trigger influence not only susceptibility, but also the severity, pathogenesis and therapy outcome.

Heterogeneity of RA is also demonstrated at the level of gene expression. Genome-wide gene expression analysis revealed evidence for molecular differences between RA patients, in particular in the type I interferon (IFN) system; Approximately 50% of RA patients (1) display a so-called "type I IFN signature" in the peripheral blood. Presence of such a signature has been described for other autoimmune diseases as well, including systemic lupus erythematosus (SLE) (2).

The type I IFN signature represents a response program that consists of genes that are activated by type I IFNs. Induction of the IFN response genes (IRG) is triggered via the activation of the JAK-STAT signalling pathway, more specifically via JAK1, TYK2, STAT1 and STAT2, and subsequent recruitment of IRF9 and formation of the ISGF3 transcription factor complex (3). Type I IFNs are typically induced upon pathogen recognition by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), in particular TLR3, TLR4, TLR7 and TLR9, and cytosolic RNA helicases like RIG-I and MDA5 (4). More recently, PRRs have been found to be activated upon binding of endogenous ligands as well, the so-called damage associated molecular patterns (DAMPs), like free intracellular proteins and molecules from the extracellular matrix (5;6).

Studies in serum from SLE patients have revealed that the type I IFN response in SLE is induced by immune complexes consisting of improperly cleared dead cell material and anti-nuclear antibodies such as anti-dsDNA and anti-snRNP (7-9). These immune complexes are able to induce IFNα via binding to FcγRIIA and subsequently to TLR7 or TLR9 (9). Increased activation of the type I IFN response in SLE is shown to be related to disease activity and severity (10). With regard to RA, however, no associations have been found between presence of a type I IFN signature and disease parameters such as disease activity or presence of rheumatoid factor and/or anti-citrullinated protein antibodies (11). It has been suggested that the type I IFN activity might be the result of a pathogen response; genome-wide expression analysis revealed that the gene expression profile in part of the RA patients resembled those of virus-infected primates (12). This patient group was mainly characterized by upregulation of TLR-related genes, which was associated with increased type I IRG expression, though not in an exclusive manner, i.e. some patients with a type I IFN signature did not show the pathogen-response profile and vice versa. Altogether, it is still unknown how the IFN response program is triggered in RA.

Despite the absence of an association with conventional disease parameters, several studies have demonstrated that the type I IFN signature in RA plays a role in RA pathology and has potential clinical relevance. In the preclinical phase of RA, a part of arthralgia patients already displays a type I IFN signature, which is shown to be associated with increased risk of developing arthritis (13;14). In later phases of the disease, presence of a type I IFN signature has been found to be associated with clinical response to different treatment regimens, such as rituximab

(15-18), tocilizumab (19) and anti-TNF therapy (20;21). These findings indicate that better understanding of the mechanism behind the activation of the type I IFN system in RA could provide insight into pathology and personalized treatment strategies.

In the present study, we aimed to gain more insight into the induction of the type I IFN response in RA. Thereto, we investigated whether serum from RA patients contains IFN-inducing components that might contribute to the type I IFN signature, in comparison with serum from SLE patients, of which the IFN inducing capacity is well-studied.

Methods

Patient and controls

RA patients (n=18) and SLE patients (n=25) were recruited from VU University medical center, Amsterdam. RA patients fulfilled the revised American College of Rheumatology (ACR) 1987 criteria for the diagnosis of RA (22). All RA patients were candidates for rituximab therapy because of their high disease activity (DAS28>3.2) despite DMARD treatment and previous anti-TNF therapy. At the moment of blood collection, patients were off anti-TNF therapy for at least four weeks and had not received their first RTX dose yet. All SLE patients fullfilled the updated revised ACR criteria for SLE (23). Because type I IFN activity in SLE is described to be related to disease activity (10), SLE patients were selected based on either low or high disease activity scores (SLEDAI=0 (n=16) or SLEDAI>6 (n=9)). The patient's characteristics are shown in **Table 1**.

Table 1 Patient characteristics

	RA cohort N=18	SLE cohort N=25
Demographics		
Age (years), mean ± SD	58 ± 9.3	44 ± 13.5
Female, n (%)	15 (83)	22 (88)
Disease characteristics		
Disease activity, mean ± SD *(RA:DAS28 score, SLE: SLEDAI)*	5.5 ± 1.2	4.52 ± 6.87 (SLEDAI 0, n=16; SLEDAI ≥ 6 n=9)
IgM-RF positive, n (%)	16 (89)	N/A
ACPA positive, n (%)	15 (83)	N/A
IgM RF and ACPA positive, n (%)	15 (83)	N/A
ANA positive, n (%)	11 (61)	19 (76)
Anti-dsDNA positive	0 (0)	7 (28)
ENA positive	1 (6)	13 (52)
Medication		
Current prednisolone use, n (%)	15 (83)	11 (44)
Current MTX use, n (%)	12 (67)	1 (4)
Current SSZ use, n (%)	3 (17)	0 (0)
Current HCQ use, n (%)	5 (28)	20 (80)
Current AZA use, n (%)	0 (0)	8 (32)

SD, standard deviation; DAS28, disease activity score of 28 joints; SLEDAI, SLE disease activity index; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibodies; MTX, methotrexate; SSZ, sulfasalazine; HCQ, hydroxychloroquine; AZA, azathioprine.

Blood collection

From all patients and healthy donors, blood was collected in a serum separation tube (SST tube, BD Biosciences, San Jose, California, USA). After clotting, serum was isolated by centrifugation, according to the manufacturer´s protocol, and as stored at -80 until further use.

In addition, blood from healthy donors was collected in heparin tubes and used for isolation of peripheral blood mononuclear cells (PBMCs). PBMCs were isolated by density gradient centrifugation using Lymphoprep (Axis Shield, Oslo, Norway), according to the manufacturer's protocol, and stored in liquid nitrogen until further use.

IRG induction assay

Healthy donor PBMCs were incubated with 25% patient serum for 4h or 8h at 37°C and 5% CO_2. Where indicated, samples were co-cultured with 2µg/ml cycloheximide (Sigma, Saint Louis, Missouri, USA). After incubation, cells were harvested, washed and lysed in RLT buffer (Qiagen Benelux BV, Venlo, The Netherlands) according to the manufacturer's protocol. The lysates were stored at -20°C until RNA isolation.

RNA was isolated from the cell lysates using the RNeasy Micro kit (Qiagen), according to the manufacturer's protocol. A DNAse (Qiagen) step was included to remove genomic DNA. Quantity and purity of the RNA was determined using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Delaware USA). 50ng of RNA was used for cDNA synthesis, which was performed using the the Revertaid H-minus cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany), according to the manufacturer's protocol.

IFN response gene (IRG) mRNA expression was measured by Quantitative RT-PCR (qPCR). qPCR was performed using SYBRGreen (Applied Biosystems, Foster City, CA, USA) and an ABI Prism7500HT Sequence detection system (Applied Biosystems), according to the manufacturer´s protocols. Primers were designed using Primer Express software and guidelines (Applied Biosystems) and are listed in **Table 2**. To calculate arbitrary values of mRNA levels and to correct for differences in primer efficiencies a standard curve was constructed. Expression levels of target genes were calculated relative to housekeeping gene 18S ribosomal RNA (18SrRNA). To correct for any variation between experiments, all expression values are shown relative to untreated. The overall IRG induction was determined by calculating the average expression of three known IRGs; RSAD2, IFI44L and MX1. To distinguish between type I and type II IFN response gene induction, we also measured ICAM1, a gene specifically induced by type II IFN and not by type I IFNs (1).

Table 2 Primers used for quantitative realtime PCR

Gene of interest	Forward sequence	Reverse sequence
18SrRNA	CCGAGTAAGTGCGGGTCATAA	CCATCCAATCGGTAGTAGCG
RSAD2	GTGGTTCCAGAATTATGGTGAGTATTT	CCACGGCCAATAAGGACATT
IFI44L	CCGAGCGGTATAGGATATATTCTGTT	TGCTCCTTCTGCCCCATCTA
MX1	TTCAGCACCTGATGGCCTATC	GTACGTCTGGAGCATGAAGAACTG
ICAM1	CACCTCGGTCCCTTCTGAGA	TGACATCCGCAAACAGAGTGA

IFNα assay

The PBMCs prepared from healthy blood donor buffy coats were incubated with 5% patient serum from patients or healthy individuals in the presence or absence of either 25% apoptotic material or 10% necrotic cell material. The optimal concentration of serum was first titrated on PBMC from two healthy donors. The apoptotic and necrotic material was prepared from U937 cells as previously described (8). Cell cultures were incubated for 20h at 37°C, 5% CO_2 and the IFNα levels in the culture supernatants were determined by an immunoassay (24). The sera were tested on two healthy donors, and the donor with the highest sensitivity to SLE IgG was selected for further analysis. Due to volume limitations, 2/18 RA samples were not included.

ANA and ENA measurements

Antinuclear antibody (ANA) detection by indirect immunofluorescence (IIF) was performed on Hep-2000 cells according to the instructions provided by the manufacturer (Immuno Concepts, Sacramento, CA). Hep-2000 cells are transfected with the gene for SSA-60, allowing SSA-antibody detection. Serum samples were screened in a 1:40 dilution. FITC-conjugated goat anti-human IgG antibody was used for detection of ANA. Five staining patterns were considered ANA positive: SSA pattern, homogenous, speckled, centromere, and nucleolar. Slides were evaluated with a fluorescent microscope (Leica DMRB, Leica Microsystems, Heerbrugg, Switzerland). All slides were evaluated by two independent observers; in case of a difference in opinion, a third observer was decisive.

ANA positive sera were subsequently analyzed for dsDNA specific antibodies and extractable nuclear antigen (ENA) specific antibodies using a commercially available FEIA (EliA, ImmunoDiagnostics, Thermo Fisher Scientific, Freiburg, Germany). The ENA screening test (ENA symphony) included SmD, SS-A/R060, R052, SS-B/La, CENP-B, Scl-70, Jo-1 and U1RNP. Cases positive in the ENA screen were tested for antibodies against the individual antigens. The assay was performed according to the manufacturer's instructions. All assay procedures were fully automated in an ImmunoCAP250 (Thermo Fisher Scientific). The reference range was supplied by the manufacturer. For dsDNA, values above 15 U/ml were considered positive (10-15 U/ml equivocal). For all other antigens, values above 10 U/ml (7-10 U/ml equivocal) were considered positive.

Statistical analyses

Based on data normality, as assessed by the Kolmogorov-Smirnov test, all analyses were performed using either student's t test or Mann Whitney U test. All analyses were performed using Graphpad Prism 5 Software.

Results

IRG inducing capacity

In order to study the ability of serum from RA and SLE patients to induce a type I IFN response, the patient sera were tested for their capacity to induce IFN response gene (IRG) expression. Healthy PBMCs were exposed to patient serum for either 4h or 8h, after which the IRG induction in these PBMCs was determined.

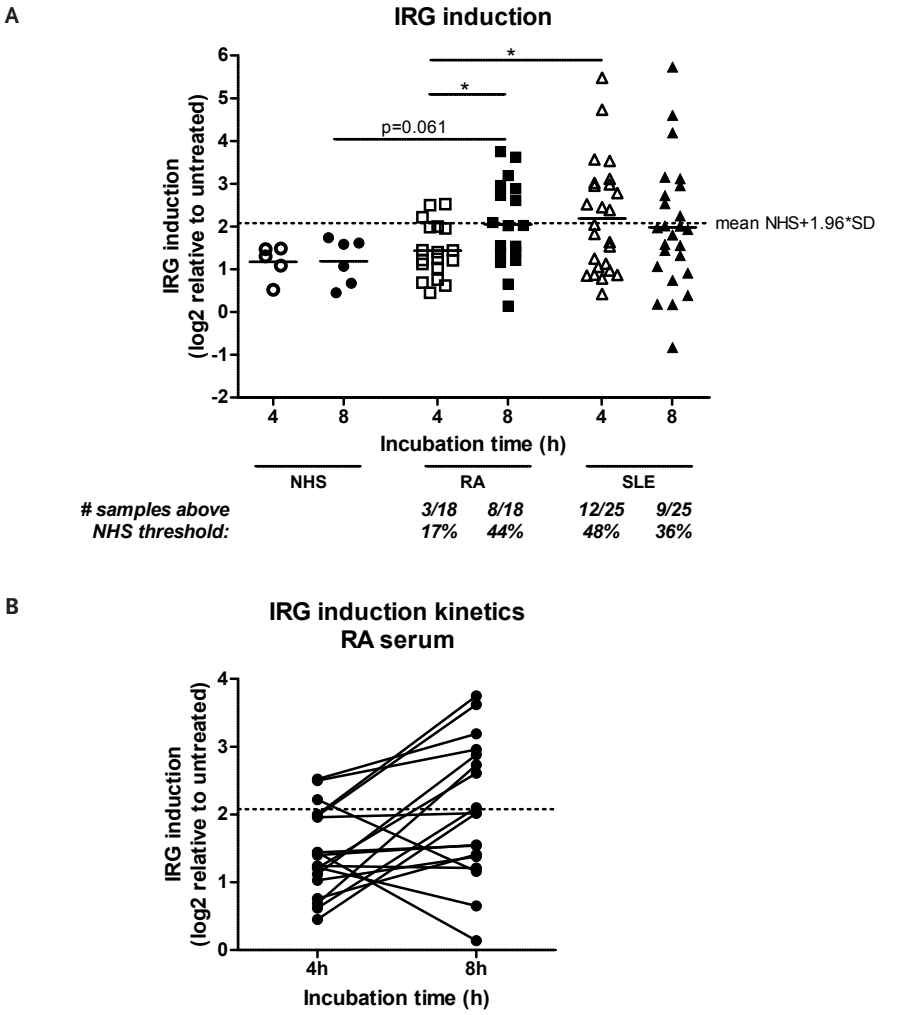


Figure 1 IRG induction capacity of patient serum. A) IRG induction by serum from SLE patients and RA patients at two different time points, compared to normal human serum (NHS) The dotted line indicates the NHS threshold, defined as the mean NHS control samples, plus 1.96 times the standard deviation (2.08) B) In time kinetics of IRG induction by RA serum.

After 4h incubation, the mean IRG induction levels in both groups were not significantly different from NHS (**Figure 1A**, RA: p=0.398, SLE: p=0.097), the mean IRG induction by SLE serum was significantly higher compared to RA serum (p=0.028). A part of the RA and SLE serum samples displayed IRG induction levels above the 95% limits of NHS (RA:3/18 samples, SLE:12/25 samples), indicating that these samples contain a rapid IRG inducing factor, such as type I IFN itself. After 8h incubation, the mean IRG induction by RA serum significantly increased compared to 4h (p=0.018), trending towards significantly higher than NHS (p=0.061, 8/18 samples above the 95% limits of NHS) and reaching levels comparable to those of SLE serum (**Figure 1A** and **1B**).

IRG induction by SLE serum after 8h incubation was not significantly different from 4h incubation. Of the 18 RA samples, 11 samples showed a more than 1.2-fold increase in IRG induction from 4h to 8h (see **Figure 1B**), compared to only 6 of the 25 SLE samples (data not shown). This suggests that part of the RA serum samples contains other types of IRG inducers than SLE serum, acting rather slow or indirect. None of the RA serum samples showed significant ICAM1 induction (data not shown), indicating that the IRG induction is specifically mediated by type I IFN activity. In line with known literature on the IFN signature in SLE and RA, the IRG induction both after 4h and 8h by SLE serum was strongly correlated to the patients' disease activity, and significantly higher in patients with a high SLEDAI compared to a low SLEDAI, but this was not observed for the IRG induction by RA serum (SLE serum: Pearson $r \geq 0.57$, $p \leq 0.003$ and SLEDAI high vs. low $p \leq 0.006$. RA serum: correlation $p \geq 0.38$).

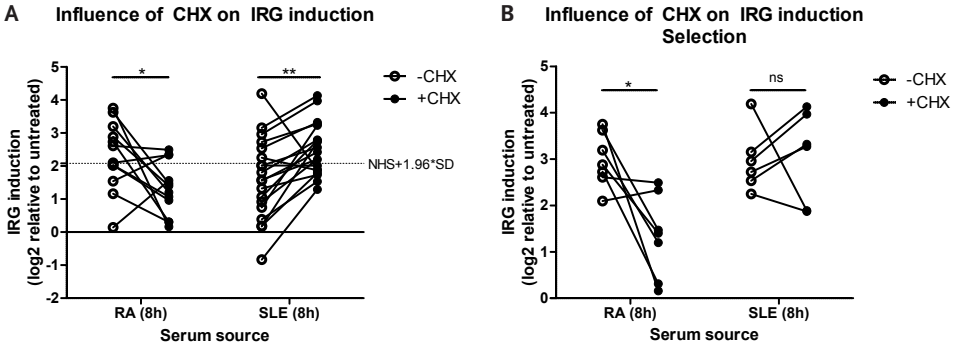


Figure 2 Dependence of the IRG induction on de novo protein synthesis. The influence of cycloheximide (CHX) on the IRG induction by patient serum. A) in all samples, B) in the selection of samples that showed IRG induction above the 95% limits of NHS when not co-cultured with CHX.

Dependency of IRG induction on *de novo* protein synthesis

To gain more information about the type of IRG inducer in the patient sera, part of the serum samples was co-cultured with the protein synthesis inhibitor cycloheximide (CHX). In PBMCs treated with RA serum, IRG induction at 8h was significantly downregulated upon CHX treatment, whereas a significant increase was observed upon CHX treatment in the cultures with SLE serum (**Figure 2A**, RA: p=0.033, SLE: p=0.002). This demonstrates that the IRG induction by RA serum, in contrast to SLE serum, depends on *de novo* protein synthesis, again indicative of an indirect IRG induction process.

IFNα protein induction capacity of RA and SLE serum

To study whether the IRG induction by patient sera is associated with the production of IFNα protein, we tested serum from RA patients and SLE patients for their capacity to induce production of IFNα in healthy donor PBMCs, using an assay that is well-established in the field of SLE (7;8).

As shown in **Figure 3A**, serum from SLE patients indeed induced significant IFNα production compared to normal human serum (NHS) ($p<0.001$); 88% of the SLE sera (22/25) induced IFNα levels above the NHS threshold. The IFNα protein production was positively correlated to the

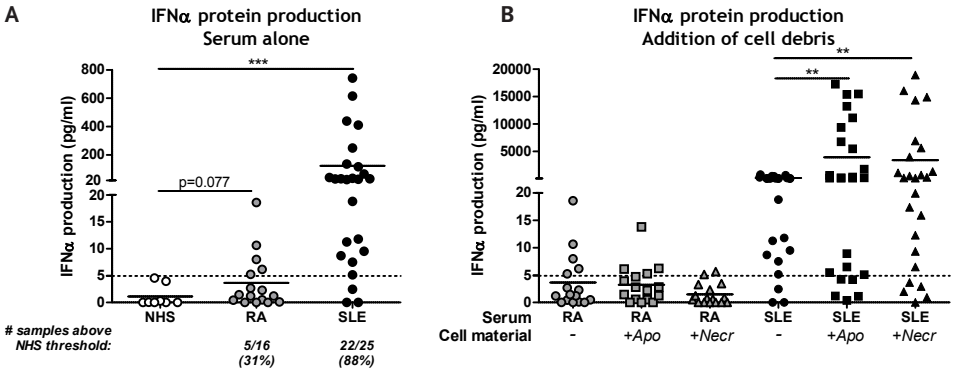


Figure 3 IFNα protein production as induced by serum from RA patients and SLE patients. A) Incubations with serum alone, without the addition of apoptotic or necrotic cell material B) Incubations with serum in the presence of apoptotic (UV-treated) or necrotic (freeze-thawed) cell material. The dotted line indicates the NHS threshold, defined as the mean NHS control samples, plus 2 times the standard deviation (4.787pg/ml).**p 0.01-0.001, ***p<0.001.

IRG induction (Spearman r=0.557, p=0.004 at 4h and r=0.665, p=0.0003 at 8h). This confirms earlier findings on the presence of an IFNα-inducing factor in serum from SLE patients (7;8). On the other hand, only 31% of the RA sera (5/16) induced IFNα protein production above the NHS threshold, and the overall levels were significantly lower as compared to the SLE sera (**Figure 3A**). This indicates that only a subset of the serum samples from RA patients contains an IFNα-inducing factor, either in low amounts or with low potency compared to SLE serum. No significant correlation was observed between IFNα protein production and IRG induction by RA serum at 4h (Spearman r=0.413, p=0.112 at 4h), and only weakly at 8h (Spearman r=0.492, p=0.049) suggesting that the IRG induction by RA serum may not be mediated exclusively by IFNα.

Effect of cell material released from apoptotic or necrotic cells

It has been previously shown that the induction of IFNα by SLE serum could be further enhanced by the addition of cell material, either from apoptotic (UV-treated) cells, or from necrotic (freeze-thawed) cells (8), probably by facilitating the formation of immune complexes between ANAs and the released cell material (25). Indeed, the IFNα induction by SLE serum was significantly increased upon the addition of either apoptotic or necrotic cell material (**Figure 3B**). However, addition of necrotic or apoptotic material did not enhance IFNα induction in PBMCs incubated with RA sera (**Figure 3B**), further supporting the hypothesis that the mechanism of IRG induction by RA serum is different from the induction by SLE serum.

Relation to antinuclear autoantibodies

It is known that the induction of IFNα by SLE serum is mediated via ANAs, such as anti-RNP and anti-dsDNA (7;9;26). To evaluate whether these antibodies could play a role in RA as well, the presence of antinuclear antibodies was determined in serum from the RA patients and SLE patients. A global ANA staining as well as dsDNA measurements and a screening assay for a selection of antigens (ENAs; SS-A, SS-B, Sm, U1RNP, CENPB, Scl70, Jo-1) were performed.

Indeed, the majority of SLE patients (16/25) were positive for anti-dsDNA, -U1RNP, -SS-A, -SS-B or a combination of those. ENA positive and/or anti-dsDNA positive samples demonstrated higher IFNα protein production than ENA/anti-dsDNA negative samples (Serum alone, p=0.002; Serum+necrotic, p=0.001; Serum+apoptotic, p<0.001), as described before (9;26).

With respect to RA, 9 of 16 patients displayed a homogeneous staining pattern on the ANA screen, which can be an indication of anti-dsDNA antibodies. However, all RA patients appeared negative for anti-dsDNA and only one patient was positive for anti-SS-A on the ENA screen. This indicates that the IRG induction by RA serum cannot be explained by the presence of these antinuclear antibodies.

Relation to other antibodies

As the IRG induction by RA serum could not be completely explained by ANA-related and/or IFNα-related processes, we studied the relation between IRG induction at 8h and other antibodies. In the RA serum samples, IgM rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), both CCP2-reactivity and reactivity to specific citrullinated peptides (see **Table 4**) and anti-RA33 (27) were determined. We did not observe a direct association between levels or positivity of any of the antibodies and the IRG induction by RA serum (p≥0.207, **Table 4**), indicating that the IRG induction is not mediated by these antibodies.

Table 4 Relation between IRG induction by RA serum and autoantibody levels and positivity.

		Relation with IRG induction, p values			
		All samples		IFNα-inducing samples excluded	
		Negative vs. Positive	Correlation with titer	Negative vs. Positive	Correlation with titer
IgM-RF		0.375	0.566	0.232	0.271
ACPA		0.659	0.240	0.432	0.125
ACPA specificities	Cit-Enolase	0.630	0.774	0.927	0.847
	Cit-Vimentim	0.315	0.349	0.170	0.230
	Cit-Fibrinogen	0.960	0.413	NA[1]	0.404
	Cit-Collagen (C1)	0.617	0.708	NA[1]	0.983
RA33		NA[1]	0.207	NA[1]	0.330

[1]*t-test could not be performed because only one patient was tested positive.*
IRG; IFN response gene, IgM-RF; IgM-Rheumatoid Factor, ACPA; anti-citrullinated protein antibodies, Cit-; citrullinated.

Discussion

In the present study, we demonstrated that serum from some RA patients contains components that are able to induce the type I IFN system. Using two types of IFN-related assays, we evaluated the interferogenic properties of RA serum in comparison with SLE serum. Whereas SLE serum samples displayed high IFNα induction and IRG induction within 4 hours of incubation, as expected, results for RA serum samples were less explicit. IRG induction by RA serum was observed in 44% of the samples only after 8 hours of incubation, and it appeared not exclusively

related to IFNα. Moreover, an association between IFNα and IRG induction and positivity for antinuclear antibodies was apparent for the SLE samples, but not for the RA samples, indicating that the type I IFN induction by RA serum occurs via another mechanism than that of SLE.

Analogies have been drawn between RA and SLE. For example, cases have been described of patients presenting symptoms as well as autoantibody profiles consistent with both SLE and RA, so-called "Rhupus syndrome" (28). Although the syndrome is relatively rare and not much is known about the type I IFN activity in these patients, presence of a type I IFN signature is very probable and likely to occur via the mechanisms described for SLE. Similar mechanisms might take place in the few RA samples that were able to induce IFNα protein and/or were ANA positive. However, this does not account for the patients that induced IRG expression after 8h of incubation but showed no IFNα protein production. In accordance with the known high heterogeneity of RA, our data indicate that the processes behind type I IFN activation by RA serum might vary between patients. The absence of a strong correlation between induction of IRG and IFNα protein by RA serum indicates potential involvement of other type I IFNs than IFNα, such as IFNβ, IFNε, IFNκ or IFNω. IFNβ is the most well-studied of those, and is suggested to play a role in RA (29). In line with our results, it has been proposed before that type I IFN activity in RA could be the result of cooperation of both IFNα and IFNβ, though with inter-individual differences in the contribution of each (30;31).

Of note, the majority of RA and SLE patients was treated with prednisone and/or hydroxychloroquine (HCQ), which are known inhibitors of the IFN response (32;33) and therefore potential disturbing factors in our assay. Due the low number of patients, we could not properly assess the effect of these treatments. However, we observed IRG induction by patient serum despite these treatments, which could imply it exceeds the potential influence of the treatment. Possibly, the treatment influence is minor because we used healthy PBMCs that were not exposed to treatment in our assay.

Both the time point of IRG induction and the sensitivity to cycloheximide treatment, which is observed for the majority of RA samples, suggest that the IRG induction by RA serum occurs via an indirect pathway that requires the induction of type I IFN protein. Typical pathways of type I IFN induction are the activation of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), in particular TLR3, TLR4, TLR7 and TLR9, and cytosolic RNA helicases like RIG-I and MDA5 (4). Increased expression of TLR-related genes has been described for RA patients who displayed an expression profile resembling that of virus-infected primates (12). Studies have demonstrated that latent viral infections are more common in autoimmune arthritis, including RA, compared to reactive arthritis, but this has not been connected to presence of an IFN signature (34). There is, however, no conclusive evidence for presence of an active (viral) infection in RA patients. As a consequence, interest has increased towards potential endogenous ligands of TLR activation in RA (5;35). In particular endogenous ligands have been described for TLR4, such as HMGB1, HSP70, Tenascin-C and S100A8/S100A9. Both TLR4 and these endogenous ligands are shown to be elevated in RA (36-39) and could be potential initiators of the observed IRG induction.

Recent literature also suggests a role for certain cytokines in the induction of type I IFNs. Of particular interest for RA is the described connection between TNFα and type I IFN. It has been described that TNFα is able to induce IFNβ via an IRF1-dependent pathway, leading to IRG expression within 8h and in a CHX-dependent manner (40). The patients in our cohort have been off anti-TNF therapy for at least 4 weeks, hence TNFα levels might have been increased since then. Moreover, Bienkowska and colleagues showed that the type I IFN signature in RA was decreased upon treatment with baminercept, an inhibitor of lymphotoxin beta and LIGHT, implying a potential role for these cytokines as well (41).

Whereas ANA and anti-dsDNA positivity in RA is relatively uncommon, presence of the conventional RA-related autoantibodies, such as antibodies against citrullinated proteins (ACPA) and rheumatoid factor (RF) occurs more frequently. In our cohort, the majority of RA patients displayed positivity for ACPA and/or RF (see **Table 1**). However, no relation was found between positivity for these antibodies, nor to their titers. This would corroborate the previously described absence of association between RF and/or ACPA and the type I IFN signature in RA (11). Larger cohorts including autoantibody negative patients and patients with positivity for only one type of antibody would allow for more in-depth analysis.

In conclusion, we demonstrated presence of a type I IFN inducing factor in serum from RA patients. Although the exact source of the type I IFN response in RA remains to be elucidated, our data indicate that the mechanism behind it is distinct from that in SLE.

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Chapter 3.2

The type I interferon signature in leukocyte subsets from peripheral blood of early arthritis patients; a major contribution by granulocytes

Tamarah D. de Jong,
Joyce Lübbers,
Samina Turk,
Saskia Vosslamber,
Elise Mantel,
Hetty J. Bontkes,
Conny J. van der Laken,
Johannes W. Bijlsma,
Dirkjan van Schaardenburg,
Cornelis L. Verweij

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Abstract

Background

The type I interferon (IFN) signature in rheumatoid arthritis (RA) has shown clinical relevance in relation to disease onset and therapy response. Identification of the cell type(s) contributing to this IFN signature could provide insight into its functional consequences. This study aimed to investigate the contribution of peripheral leukocyte subsets to the IFN signature in early arthritis.

Methods

Blood was collected from 26 early arthritis patients and lysed directly or separated into peripheral blood mononuclear cells (PBMCs) and polymorphonuclear granulocytes (PMNs). PBMCs were sorted into $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells and $CD14^+$ monocytes by flow cytometry. mRNA expression of three IFN response genes (IRGs; RSAD2, IFI44L and MX1) and type I interferon receptors (IFNAR1 and IFNAR2) was determined in whole blood and blood cell subsets by qPCR. IRG expression was averaged to calculate an IFN score for each sample.

Results

Patients were designated "IFN^{high}" (n=8) and "IFN^{low}" (n=18) based on the IFN score cutoff in whole peripheral blood from healthy controls. The difference in IFN score between IFN^{high} and IFN^{low} patients was remarkably large for the PMN fraction (mean 25-fold) compared to the other subsets (mean 6-9-fold), indicating that PMNs are the main inducers of IRGs. Moreover, the relative contribution of the PMN fraction to the whole blood IFN score was 3-fold higher than expected from its abundance in blood ($p=0.008$), whereas this was 3-6-fold lower for the other subsets ($p\leq0.063$), implying that the PMNs are most sensitive to IFN signaling. Concordantly, IFNAR1 and IFNAR2 were upregulated compared to healthy controls selectively in patient PMNs ($p\leq0.0077$) but not in the PBMCs.

Conclusions

Polymorphonuclear granulocytes are the main contributors to the whole blood type I IFN signature in early arthritis patients, which seems due to increased sensitivity of these cells to type I IFN signaling. Considering the well-established role of neutrophils in the pathology of arthritis, this suggests a role of type I IFN activity in the disease as well.

Introduction

Rheumatoid arthritis (RA) manifests itself as a heterogeneous disease with a clinical spectrum ranging from mild to severe disease. This heterogeneity most likely has its origin in the multifactorial nature of the disease, whereby specific combinations of genetic risk factors together with an appropriate environmental trigger influence not only susceptibility, but also the severity, pathogenesis and therapy outcome.

Heterogeneity of RA is partly reflected at the level of gene expression. Genome-wide gene expression analysis revealed evidence for molecular differences between RA patients, in particular in the type I interferon (IFN) response gene program (1). Part of the RA patients display a so-called "IFN signature", which is characterized by relatively high expression of type I IFN response genes (IRGs). Induction of these IRGs is triggered via the activation of the type I IFN receptor, IFNAR1 and IFNAR2, which dimerize and subsequently activate the JAK-STAT signaling pathway, more specifically JAK1, TYK2, STAT1 and STAT2, eventually resulting in recruitment of IRF9 and formation of the ISGF3 transcription factor complex (2). Although the presence of the IFN signature in RA is not found to be associated with disease parameters such as disease activity or presence of rheumatoid factor and/or anti-citrullinated protein antibodies (3), several studies have demonstrated that the IFN signature in RA does have potential clinical relevance.

Presence of the IFN signature was shown to be a risk factor for arthritis development in preclinical disease (4;5). In later phases of the disease, presence of an IFN signature was found to be associated with clinical response to different treatment regimens, such as rituximab (6-8) and tocilizumab (9). Furthermore, type I IRG expression appears to be differentially regulated between responders and non-responders during treatment with rituximab and anti-TNF therapy (10-12).

Peripheral blood is an easily accessible source for biomarker identification and the studies mentioned above demonstrate that the peripheral blood from RA patients reflects pathogenic processes related to the disease. However, the peripheral blood consists of several cell types and consequently the transcriptomic profile is an accumulation of all gene expression programs that are induced in these cell types. Identification of the cell type(s) contributing to the IFN signature could provide insight into its functional consequences and potentially into personalized treatment strategies . The present study aimed to investigate the contribution of the major leukocyte subsets to the IFN signature in whole blood from early arthritis patients. Using this patient group allowed us to study the IFN signature without interference of treatment with immune-modulatory drugs that are known to affect type I IFN signaling, such as glucocorticoids or hydroxychloroquine (13-15).

Methods

Patient recruitment and blood collection

Patients (n=26) were consecutively recruited from the early arthritis cohort (EAC) within the Amsterdam Rheumatology and immunology Center, location Reade, Amsterdam, the Netherlands. Inclusion criteria were presence of ≥ 1 arthritic joint, disease duration <6 months and no previous use of DMARDS or biologicals. The majority of patients (81%) fulfilled the 2010

American College of Rheumatology criteria for the classification of RA (16). The remaining 5 patients were diagnosed with seronegative rheumatoid arthritis (n=4) and monoarthritis (n=1), according to the reumatologist's assessment. Healthy controls (HC, n=25) were recruited at the VU University medical center, Amsterdam. From each donor, approximately 20ml blood was collected by venipuncture into heparin tubes and a PAXgene tube (PreAnalytix, GmbH, Germany). The PAXgene tube was stored at -20°C until further processing. The heparinized blood was processed on the same day it was drawn. This study was approved by the medical ethics committee of VU university medical center and Reade, Amsterdam, the Netherlands and informed consent was obtained from all donors.

PBMC isolation and PMN isolation

PBMCs were isolated from heparinized blood by density gradient centrifugation using Lymphoprep (Axis Shield, Oslo, Norway), according to the manufacturer's protocol. PBMCs were washed and a minimum of $1x10^6$ PBMCs was directly lysed in 350µl RLT buffer (Qiagen Benelux BV, Venlo, The Netherlands). A minimum of $7x10^6$ PBMCs was resuspended in PBS containing 1% BSA for subsequent flow cytometric cell sorting. Polymorphonuclear (PMN) leukocytes (granulocytes) were isolated from the remaining erythrocyte/PMN pellet by lysing the erythrocytes with EL buffer (Qiagen Benelux BV, Venlo, The Netherlands) as described before (17;18), according to the manufacturer's protocol. The remaining PMN-enriched pellet was washed with PBS and lysed in 350µl RLT buffer. RLT lysates were stored at -20°C until RNA isolation.

Flow cytometry and cell sorting

Absolute number and percentage of monocytes and lymphocyte subsets were determined using flow cytometry (FACSCalibur) on whole heparinized blood. Quantification beads (Trucount, BD, San Jose, USA) in combination with fluorescein isothiocyanate (FITC)-conjugated anti-CD45, phycoerythrin (PE)-conjugated anti-CD14, peridinin chlorophyll (PerCP)-conjugated anti-CD3 and allophycocyanin (APC)-conjugated anti-CD19 were used to measure absolute number of lymphocytes, monocytes, T cells and B cells, according to the manufacturer's instructions (all from BD, San Jose, USA). For the T cell subsets anti-CD45 and anti-CD3 were taken along combined with PE-conjugated anti-CD8 and APC-conjugated anti-CD4 (all from BD, San Jose, USA).

The following antibodies were used for the cell sorting procedure (all from BD, San Jose, USA): Pacific blue-conjugated or Horizon™ V450-conjugated anti-CD3, PE-conjugated anti-CD4, FITC-conjugated anti-CD8, APC-conjugated anti-CD19, and PerCP-conjugated anti-CD14. Labeled cells were analyzed and separated using FACS Aria and FACS DIVA software 6.1.3 (Becton Dickinson, San Jose, USA). The nozzle size was 70 µm and sorting speed of 3000-5000 cells/s. For sorting purposes, a gate was set around lymphocytes and subsequent gates were set for $CD3^+CD4^+$ T helper Cells, $CD3^+CD8^+$ cytotoxic T-cells and $CD19^+$ B-cells, based on positivity of the markers. Monocytes were gated based on forward and side scatter properties as well as CD14 positivity. From each population, a minimum of 3x105 cells was sorted and subsequently spun down at 400g for 5 minutes, lysed in 350µl RLT and stored at -20°C until RNA isolation. Sorting purity was >90% for 95/104 sorted samples. Three samples, two CD19-enriched fractions and one CD14-enriched fraction were excluded due to purities below 80%.

RNA isolation and cDNA synthesis

RNA was isolated from the cell lysates and PAXgene tubes using the RNeasy Micro or Mini kit (Qiagen Benelux BV, Venlo, The Netherlands) or PAXgene RNA isolation kit (PreAnalytix, GmbH, Germany), respectively, according to the manufacturers' protocols. In both procedures, a DNAse (Qiagen Benelux BV, Venlo, The Netherlands) step was included to remove any genomic DNA. RNA quantity and purity were determined using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, USA). Either 50 ng (cell fractions) or 250 ng (PAXgene whole blood) RNA was used for cDNA synthesis, which was performed using the a Revertaid H-minus cDNA synthesis kit (Thermo Scientific, Waltham, USA), according to the manufacturer's protocol. Two CD19-enriched sample were excluded because of low RNA yield.

qPCR and calculation of the IFN score

We determined the mRNA expression of three IFN response genes (IRGs) (IFI44L, MX1 and RSAD2) that are described to be components of the IFN signature in RA (1;7;10), and thus believed to reflect the type I IFN response in peripheral blood. IRG mRNA expression was measured on cDNA by quantitative PCR (qPCR). qPCR was performed using SYBR Green (Applied Biosystems, Foster City, USA) and an ABI Prism7500HT Sequence detection system (Applied Biosystems, Foster City, USA), according to the manufacturer´s protocols. Primers were designed using Primer Express software and guidelines (Applied Biosystems, Foster City, USA) and are listed in Table S1 (Supplementary material). To calculate arbitrary values of mRNA levels and to correct for differences in primer efficiencies a standard curve was constructed. Expression levels of target genes were calculated relative to housekeeping gene GAPDH. Expression levels of the IRGs were highly correlative for all studied cell fractions ($r \geq 0.708$, $p < 0.0001$), therefore an IFN score was calculated by averaging the expression levels of all 3 genes for each sample. Presence of a type I IFN signature (referred to as IFN^{high}) was defined as an IFN score above mean + 2*SD in healthy controls. Each IRG was also analyzed individually, which yielded comparable results as described below (data not shown).

Statistical analysis and calculation of expected and observed contributions

All analyses were performed using Mann Whitney U tests in Graphpad Prism 5 Software. In order to study the relative contribution of each cell type to the whole blood IFN signature, we calculated an "expected" and "observed" IFN score contribution. The "expected" contribution is based only on the distribution of the cell types in the blood and assumes that each cell type would contribute equally to the whole blood IFN signature. E.g. for a whole blood sample with an IFN score of 2.5 which contained 3.3% monocytes, the expected contribution of the monocytes would be 2.5 * 0.033 = 0.0825. The "observed" contribution is the IFN score as it was measured in a sorted cell subset, corrected for the abundance of this subset in whole blood. E.g. if the sample described above had an IFN score of 3.5 in the CD14-enriched fraction, the observed IFN score contribution of the monocytes is 3.5 * 0.033 = 0.1155.

Results

Patients characteristics and selection of IFN^{high} and IFN^{low} patients

First, patients were separated into an IFN^{high} and IFN^{low} group based on their IFN score in whole blood. As displayed in **Figure 1**, the IFN signature was present in 8/26 patients, these will be referred to as "IFN^{high}", and the remaining 18 patients were designated "IFN^{low}". Patient characteristics are shown in **Table 1**. The IFN^{high} group displayed slightly shorter duration of symptoms and a higher percentage of ACPA positive patients, but this did not reach statistical significance (Symptom duration p=0.137, ACPA positivity p=0.084 (Fisher's exact)).

General abundance of cell subsets in relation to the whole blood type I IFN profile

In order to gain insight into the cell subset composition of the peripheral blood in relation to the presence of the IFN signature, we compared the number of total $CD3^+$ T cells, $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, $CD19^+$ B cells, $CD14^+$ monocytes, and granulocytes (PMNs) between

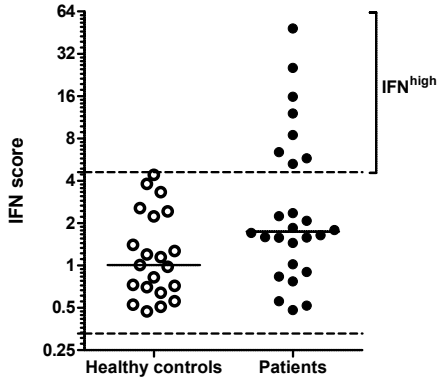

Figure 1 Whole blood IFN scores in all 26 early arthritis patients. Eight patients were designated IFN^{high} based on the mean+2SD cutoff in healthy controls. Patients within the 95% limits of healthy controls (indicated between the dashed lines) were considered IFN^{low}.

Table 1 Patient characteristics

	Healthy controls	All patients	IFN^{low}	IFN^{high}
N	25	26	18	8
Female, n (%)	16 (64)	20 (77)	13 (72)	7 (88)
Age in years, mean (SD)	35 (10)	47 (14)	48 (16)	44 (9)
DAS28, mean (SD)	n/a	4.6 (1.2)	4.7 (1.3)	4.4 (1.0)
Duration of symptoms in weeks, mean (SD)†	n/a	16 (25)	20 (29)	8 (8)
IgM RF positivity, n (%)	n/a	19 (73)	13 (72)	6 (75)
ACPA positivity, n (%)	n/a	15 (58)	8 (44)	7 (88)

†Data missing for 1 patient.
SD, standard deviation; IgM RF, IgM rheumatoid factor; ACPA, anti-citrullinated protein antibodies

IFN^{high} and IFN^{low} patients. As shown in **Table 2**, we observed a tendency towards lower numbers of all lymphocyte subsets in IFN^{high} patients compared to IFN^{low} patients, but this did not reach statistical significance (p≥0.07). Remarkably, only the number of PMNs was significantly higher in IFN^{high} patients compared to IFN^{low} patients, (1.6-fold, p=0.031). The cell percentages also displayed a slightly higher PMN percentage and lower lymphocyte percentage in IFN^{high} patients compared to IFN^{low} patients, although this was not significant (**Table 2**). The fold difference we observed in whole blood IFN score between IFN^{low} patients and IFN^{high} patients (12-fold) greatly exceeded the fold difference observed in PMN abundance (1.6-fold), indicating that the presence of the IFN signature in these patients is not primarily caused by predominance of a particular cell subset.

Table 2 Abundance of leukocytes and subsets in patient whole blood.

	Based on	All patients	IFN^{low}	IFN^{high}	Fold difference between means	Comparison of means, p value
Total leukocytes	CD45$^+$	7191 ± 2626	7271 ± 2981	7020 ± 1814	1.04 (low > high)	0.798
Lymphocytes	CD45$^+$, FSC/SSC	1856 ± 481 (27.8% ± 9.7)	1955 ± 478 (29.7% ± 10.6)	1646 ± 442 (24.0% ± 6.1)	1.19 (low > high)	0.194
T cells	CD3$^+$	1349 ± 365 (20.2% ± 7.2)	1441 ± 363 (21.8% ± 7.8)	1153 ± 304 (16.9% ± 4.6)	1.25 (low > high)	0.066
Helper T cells	CD3$^+$, CD4$^+$	850 ± 228 (13.1% ± 5.0)	899 ± 214 (13.8% ± 5.3)	729 ± 229 (11.6% ± 4.1)	1.23 (low > high)	0.130
Cytotoxic T cells	CD3$^+$, CD8$^+$	465 ± 232 (6.9% ± 3.4)	514 ± 245 (7.6% ± 3.7)	345 ± 148 (5.2% ± 1.9)	1.49 (low > high)	0.187
B cells	CD19$^+$	268 ± 125 (4.0% ± 1.9)	284 ± 140 (4.2% ± 2.1)	234 ± 81 (3.5% ± 1.3)	1.21 (low > high)	0.549
Monocytes	CD14$^+$	336 ± 127 (4.8% ± 1.5)	353 ± 140 (5.0% ± 1.4)	299 ± 92 (4.4% ± 1.5)	1.18 (low > high)	0.406
PMNs	FSC/SSC	3757 ± 2715 (67.4% ± 10.1)	3137 ± 2945 (65.3% ± 11.0)	5075 ± 1598 (71.6% ± 6.7)	1.61 (low < high)	0.031

FSC, forward scatter; SSC, side scatter. Cell amounts are indicated in numbers per µl, mean ± standard deviation. Percentages of total leukocytes are indicated between brackets, mean ± standard deviation.

Contribution of sorted cell subsets to the IFN score

Next, we compared the contribution of individual leukocyte subsets to the IFN signature. As shown in **Figure 2**, IFN scores were significantly different between IFN^{high} and IFN^{low} patients for all cell subsets, which is to be expected as all cell types presumably possess type I IFN signaling ability. The difference between IFN^{high} and IFN^{low} patients was most prominent for the PMN fraction, which displayed a 25-fold higher mean IFN score in IFN^{high} patients compared to IFN^{low} patients (p<0.0001, **Figure 2**). These measurements are normalized on RNA input and the expression levels are relative to the housekeeping gene GAPDH, hence these data are irrespective of cell abundance.

In order to investigate the relative contribution of the leukocyte subsets in relation to their distribution in peripheral blood, we used the expression data in whole blood from IFN^{high} patients and the relative abundance of each subset to estimate an "expected" cell subset contributions, assuming that each subset would contribute equally to the IFN score. Subsequently, we compared

the estimated cell subset contributions to the actual contributions as measured in the sorted cell subsets, corrected for its abundance ("observed" contribution). Details about the calculation of the expected and observed contributions are described in the methods section.

As shown in **Table 3**, all cell types showed a difference between the observed contributions and the expected contributions to the IFN score. The observed contributions of $CD4^+$ Helper T cells, $CD8^+$ cytotoxic T cells, $CD19^+$ B cells and $CD14^+$ monocytes were 2.8-6.3-fold lower than the expected contributions, which was significant for most subsets ($p \leq 0.0625$). Remarkably, the observed contribution of the PMNs was 3.4-fold higher than its expected contribution ($p=0.0078$). This tendency remained present after correction for any differences in RNA yield between subsets (data not shown). The sum of the RNA-corrected observed contributions of all cell subsets per patient was somewhat higher than the total IFN score as measured in whole blood (mean difference 1.2-fold, not significantly different). This could be explained by slight impurities in each isolated subset, and implies that there is no other cell population substantially contributing to the whole blood IFN score, as this would have resulted in a lower sum compared to the whole blood IFN score. Altogether, these data indicate that PMNs are the main contributors to the whole blood IFN score, not only because of its high abundance in whole blood, but also because of an increased potency to induce IRGs.

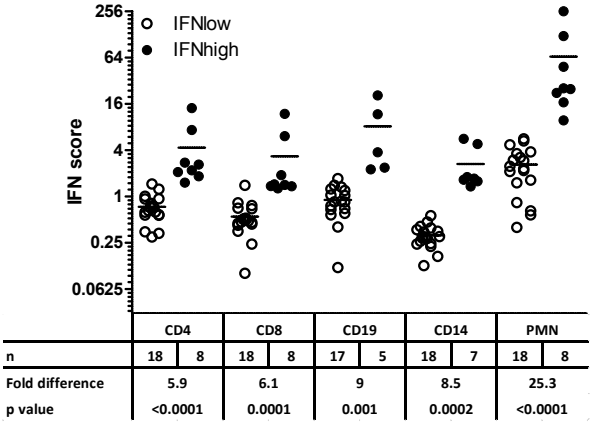

Figure 2 IFN scores per leukocyte subset of IFN^{low} and IFN^{high} patients. Fold differences between the two groups, as well as p values of the statistical comparisons, are indicated below the graph.

Table 3 Expected and observed contributions of leukocyte subsets to the IFN score in whole blood of IFN^{high} patients.

	Expected	Observed	Direction	Mean fold difference	P value
CD4	1.843 ± 1.715	0.521 ± 0.507	Exp > Obs	3.94 ± 1.71	0.0156
CD8	0.741 ± 0.504	0.156 ± 0.127	Exp > Obs	5.36 ± 2.90	0.0223
CD19	0.530 ± 0.499	0.298 ± 0.263	Exp > Obs	2.78 ± 0.68	0.0625
CD14	0.679 ± 0.594	0.126 ± 0.110	Exp > Obs	6.25 ± 2.53	0.0156
PMNs	11.71 ± 11.53	48.33 ± 65.89	Exp < Obs	3.35 ± 1.29	0.0078

Relation between IFN score and type I IFN receptor expression in subsets and whole blood

The data described above suggests an increased sensitivity of IFN^{high} PMNs to type I IFNs. To gain more insight into the mechanism behind this increased sensitivity, we measured the mRNA expression of the upstream receptors of type I IFN signaling, i.e. IFNAR1 and IFNAR2. Although we observed a correlation between the subset's IFN score and IFNAR1 expression for all subsets, the correlation between the subset's IFN score and IFNAR2 expression was only significant for the PMN fraction (Spearman r: 0.461, p=0.020, **Table 4**). Furthermore, both IFNAR1 and IFNAR2 expression was highest in the PMN fraction compared to the other fractions, indicating that PMNs could be more sensitive to type I IFN binding.

Table 4 IFNAR1 and IFNAR2 expression and the relation to the subset IFN scores

	Average expression ± SD		Correlation with subset's IFN score	
IFNAR1	**All patients**	**IFN^{high} patients**	**Spearman r**	**p value**
CD4	2.473 ± 1.157	3.440 ± 1.309	0.679	0.0001
CD8	1.723 ± 0.701	1.976 ± 0.730	0.363	0.069
CD19	3.687 ± 1.756	3.844 ± 1.487	0.525	0.012
CD14	0.687 ± 0.294	0.908 ± 0.354	0.371	0.068
PMN	5.089 ± 2.243	6.529 ± 1.649	0.461	0.020
IFNAR2	**All patients**	**IFN^{high} patients**	**Spearman r**	**p value**
CD4	4.544 ± 3.247	3.531 ± 1.234	0.179	0.382
CD8	3.952 ± 2.736	2.969 ± 0.994	-0.037	0.857
CD19	4.213 ± 2.389	2.978 ± 0.908	-0.03	0.895
CD14	0.946 ± 0.562	0.748 ± 0.304	0.042	0.842
PMN	5.749 ± 5.039	6.231 ± 3.340	0.507	0.010

Specific upregulation of type I IFN receptors in early arthritis PMNs

Since the PMN fraction showed high activation of the IFN response that appeared to be related to expression of the type I IFN receptors, we compared IFNAR1 and IFNAR2 mRNA expression in isolated PBMCs and PMNs of patients to those of healthy controls. As shown in **Figure 3**, we observed no differences in expression of IFNAR1 or IFNAR2 in the PBMC fractions of patients compared to healthy control PBMCs (p=0.387 and p=0.902, respectively). However, both IFNAR1 and IFNAR2 expression were considerably increased in the PMN fraction of patients compared to healthy control PMNs (IFNAR1 3.0-fold, p<0.001, IFNAR2 2.5-fold, p=0.008). Only IFNAR1 expression was significantly different between IFN^{low} and IFN^{high} patients (p=0.021, see **Figure 3A**), implying that the extend of the IFN signature might not solely depend on IFNAR expression.

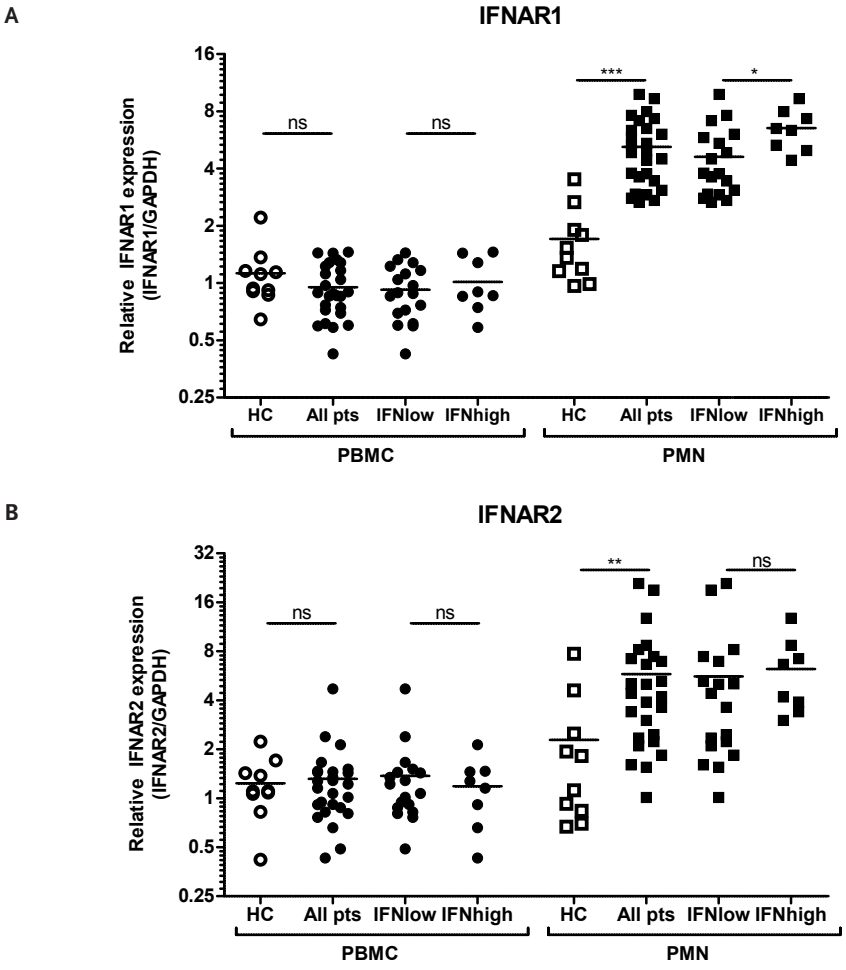

Figure 3 Selective upregulation of IFNAR expression in PMNs from early arthritis patients. IFNAR1 (A) and IFNAR2 (B) expression in PBMCs and PMNs from healthy controls and early arthritis patients. ** p 0.01 - 0.001; *** p≤0.001.

Discussion

The type I IFN signature in peripheral blood from RA patients was first described in 2007 (1) and since then, it has been extensively studied in relation to disease onset and therapy response. Occasionally, IFN response gene (IRG) expression in RA was assessed in isolated cell subsets instead of whole blood, such as PBMCs (6;19), monocytes (20) or neutrophils (21). The present study is the first to demonstrate that there is diversity in the contribution of whole blood cell subsets to the extent of the type I IFN response, with a major contribution by PMNs.

Patients with an IFN signature (IFN^{high}) did not appear clinically different from patients without this signature (IFN^{low}). Although our cohort is rather small, this corroborates previous studies

(1;3). We observed slightly lower lymphocyte counts and slightly higher neutrophil counts in IFN^{high} patients compared to IFN^{low} patients, but these differences were too small to fully explain the difference in whole blood IFN score between the two groups. Concordantly, our data suggest that the whole blood IFN signature is facilitated by a selective change in PMN sensitivity to type I IFN signaling rather than by a great difference in cell abundance.

The PMN fraction primarily consists of neutrophils, which have been shown to play a role in RA. They are the first cells to enter the joint when the disease starts and are the most abundant cell type present in the joint (22;23). Neutrophils in the RA joint display a "primed" phenotype compared to control neutrophils, resulting in increased cytokine and chemokine production, decreased apoptosis rates (24), the gained ability to present antigens (25) and upregulation of chemokine receptors to induce migration of other immune cells (26).

We observed that patient PMNs, but not the PBMCs, displayed type I IFN receptor (IFNAR1 and IFNAR2) upregulation compared to healthy controls, which was not completely dependent on the presence of the IFN signature. It has been suggested that RA neutrophils would mainly become primed and activated within the inflamed joint due to the large amount of cytokines present. However, the IFNAR1 and IFNAR2 upregulation in the circulating PMNs suggests that these cells could also have gained a primed phenotype. Wright and colleagues have described the gene profiles that are induced upon neutrophil priming with TNFα or GM-CSF, which did not involve upregulation of IFNAR1, and even seemed to cause downregulation of IFNAR2 (27). Broader gene expression and protein expression studies on RA PMNs, possibly paired with synovial PMNs are required to gain more insight into the exact gene profile and source of the priming.

It was demonstrated that healthy mature neutrophils already display increased expression of IFNAR1 and IFNAR2 as well as type I IFN response genes compared to immature neutrophils (28). Of interest, these mature neutrophils were more prone to IFNα-mediated induction of neutrophil extracellular trap (NET) formation than immature neutrophils. NETs are extracellular structures that consist of chromatin and neutrophil-related proteins and are released by neutrophils under (auto-)inflammatory conditions. Neutrophils from RA blood and synovial fluid are shown to exhibit increased spontaneous NET formation compared to neutrophils from healthy controls (29;30) or osteoarthritis patients (31). A study in SLE patients demonstrated that NETs contain a considerable source of type I IFN-inducing agents (32). Altogether, the upregulation of IFNAR1 and IFNAR2 we observed in RA PMNs, together with the increased spontaneous NET formation, could contribute to a positive feedback loop of subsequent NET-mediated type I IFN production, type I IFN binding and simultaneous IRG induction and more NET formation.

It has recently been demonstrated that the baseline IFN signature in RA PMNs is associated with a good response to anti-TNF therapy (21). Notably, earlier studies using gene expression profiling in whole blood only described a relation between IFN response regulation and therapy response during anti-TNF treatment and not between the extent of the IFN response and anti-TNF response prior to the start of therapy (11;12). Although the studies describe different types of anti-TNF treatment and the PMN findings need validation in independent studies, one could

speculate that the PMN fraction is a more homogeneous source than whole blood to study the IFN signature in relation to anti-TNF response. Moreover, neutrophils are known to both bind and secrete TNFα, and multiple studies have demonstrated that TNFα and type I IFNs might influence each other's signaling activities (33;34). Consequently, the IFN signature in PMNs might be a direct reflection of high TNFα activity and therefore indicate increased sensitivity to TNFα inhibition, ultimately resulting in a good response to therapy.

Presence of a baseline IFN signature is also shown to be associated with a poor response to rituximab treatment (6;7). The exact mechanism behind this association remains to be elucidated, but it could indicate that patients with an IFN signature have a neutrophil-dominated pathology, hence B cell depletion would have less effect on the disease activity than in IFN^{low} patients. Recently, it has been shown that rituximab treatment could lead to late-onset neutropenia in a small proportion of patients (35). It would be interesting to study this in relation to the previously reported association of rituximab-related pharmacodynamics of type I IFN response gene expression and clinical response to rituximab (10).

Considering these previously described findings regarding the IFN signature in relation to therapy response, we hypothesize that patients with an IFN signature in the neutrophils might benefit from therapies that target the activity of neutrophil-derived cytokines, such as anti-TNF therapy (21) or tocilizumab therapy (9), whereas patients without an IFN signature might benefit from rituximab therapy instead (6;7). However, more studies on the exact role of the IFN signature in neutrophil-related RA pathology are required to support this hypothesis.

Since the PMNs are considered one of the first cell types to enter the joint (22), and the presence of an IFN signature has been associated with an increased risk to develop arthritis (4;5), this could indicate that the neutrophils have been primed and activated to migrate towards the joint in order to inflict the first damage. Moreover, it could suggest that patients without an IFN signature that develop arthritis might have another mechanism behind the disease onset, e.g. mediated by B cell migration (4;36-38). Extending the present study to the preclinical phase of arthritis could give more insight into the role of the IFN signature and neutrophils in disease onset.

Conclusions

Conclusively, we have demonstrated that PMNs are the main contributors to the whole blood IFN signature in early arthritis patients. Considering the well-established role of neutrophils in the pathology of arthritis, this suggests a role of type I IFN activity in the disease as well.

Acknowledgements

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Supplementary material

Table S1 Primers used for quantitative PCR

Gene of interest	Forward primer	Reverse primer
GAPDH	GCCAGCCGAGCCACATC	TGACCAGGCGCCCAATAC
RSAD2	GTGGTTCCAGAATTATGGTGAGTATTT	CCACGGCCAATAAGGACATT
IFI44L	CCGAGCGGTATAGGATATATTCTGTT	TGCTCCTTCTGCCCCATCTA
MX1	TTCAGCACCTGATGGCCTATC	GTACGTCTGGAGCATGAAGAACTG
IFNAR1	AAAATTGTCTGGGTGTCAGAATATTACTAG	ACCAATCTGAGCTTTGCGAAA
IFNAR2	TGTATACAATCATGAGTAAACCAGAAGATTT	TTGTGTTCCCGCTGAATCCT

Chapter 3.3

Physiological evidence for diversification of IFNα- and IFNβ-mediated response programs in different autoimmune diseases

Tamarah D. de Jong*, Saskia Vosslamber*, Elise Mantel, Sander de Ridder, John G. Wesseling, Tineke C.T.M. van der Pouw Kraan, Cyra Leurs, Harald Hegen, Florian Deisenhammer, Joep Killestein, Ingrid E. Lundberg, Jiri Vencovsky, Mike T. Nurmohamed, Dirkjan van Schaardenburg, Irene E.M. Bultink, Alexandre E. Voskuyl, D. Michiel Pegtel, Conny J. van der Laken, Johannes W.J. Bijlsma, Cornelis L. Verweij

* both authors contributed equally

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Abstract

Introduction

Activation of the type I IFN response program is described for several autoimmune diseases, including systemic lupus erythematosus (SLE), multiple sclerosis (MS), myositis (IIM) and rheumatoid arthritis (RA).While IFNα contributes to SLE pathology, IFNβ therapy is often beneficial in MS, implying different immunoregulatory roles for these IFNs. This study was aimed to investigate potential diversification of IFNα-and IFNβ-mediated response programs in autoimmune diseases.

Methods

Peripheral blood gene expression of 23 prototypical type I IFN response genes (IRGs) was determined in 54 healthy controls (HCs), 69 SLE (47 test, 22 validation), 149 IFNβ-treated MS (71 test, 78 validation), 160 untreated MS, 78 IIM and 76 RA patients. Patients with a type I IFN signature were selected for analysis.

Results

We identified IFNα- and IFNβ-specific response programs (GC-A and GC-B, respectively) in SLE and IFNβ-treated MS patients. Concordantly, the GC-A/GC-B log-ratio was positive for all SLE patients and negative for virtually all IFNβ-treated MS patients, which was confirmed in additional cohorts. Applying this information to other autoimmune diseases, IIM patients displayed positive GC-A/GC-B log-ratios, indicating predominant IFNα activity. The GC-A/GC-B log-ratio in RA was lower and approached zero in part of the patients, implying relative importance of both clusters. Remarkably, GC-A/GC-B log-ratios appeared most heterogeneous in untreated MS; half of the patients displayed GC-A dominance, whereas others showed GC-B dominance or log-ratios near zero.

Conclusion

Our findings show diversification of the type I IFN response in autoimmune diseases, suggesting different pathogenic roles of the type I IFNs.

Type I interferons (IFNs) comprise a large family of cytokines with antiviral, immunomodulatory and anti-proliferative activities. The type I IFN family consists of 17 closely related members, including 13 IFNα subtypes and 4 unique members, i.e. IFNβ, IFNε, IFNκ and IFNω, of which IFNα and IFNβ are most commonly expressed and well-characterized.

Type I IFNs achieve their biological effects by binding to multi-subunit receptors, IFNAR1 and IFNAR2, on the cell surface. This leads to receptor dimerization and activation of the JAK-STAT pathway, a complex cascade of intracellular secondary messengers that emerge in transcriptional activation of genes containing IFN-stimulated response elements (ISRE) and/or IFN gamma-activated sequence response elements (GAS) (1-4). Upregulation of type I IFN response genes (IRGs) is referred to as a "type I IFN signature" and is a reflection of type I IFN bioactivity.

Initially, type I IFNs were defined by their antiviral effects and as a consequence, they were used for the treatment of chronic viral infections such as hepatitis B and hepatitis C (5). The antiviral activity involves suppression of viral replication, induction of apoptosis in virally infected cells, stimulation of T cell and B cell responses, natural killer cell-mediated and $CD8^+$ T cell-mediated cytotoxicity and activation of dendritic cells (DCs) (6).

Increasing insight in the activities of type I IFNs revealed their role as pleiotropic cytokines with a critical role in modulating immune responses. Several observations indicated involvement of type I IFNs and the presence of a type I IFN signature in autoimmune diseases including systemic lupus erythematosus (SLE), Sjögren´s syndrome, systemic sclerosis, multiple sclerosis (MS), idiopathic inflammatory myopathies (IIM) and rheumatoid arthritis (RA) (7-9). Compelling evidence from studies in SLE demonstrated that particularly IFNα is directly implicated in its pathogenesis (10;11). SLE is characterized by the presence of autoantibodies to nucleic acid and associated proteins, which are able to induce IFNα protein (12). Serum levels of IFNα are increased in SLE and associated with disease severity and organ involvement (13;14). Supportive for a pathogenic role of IFNα in SLE was the observation that virally infected people and cancer patients who were treated with IFNα sometimes produced anti-nuclear antibodies and occasionally developed SLE-like symptoms (15;16). The mechanisms by which IFNα may contribute to autoimmunity are the induction of autoreactive lymphocytes, enhancement of long-term antibody responses and priming of myeloid cells.

In contrast to the pathogenic effects of prolonged IFNα signaling in SLE, IFNβ administration has notable therapeutic effects in MS, an autoimmune disease of the central nervous system characterized by progressive neurological dysfunction due to demyelination and axonal damage (17). In MS patients, treatment with IFNβ reduces clinical relapses and brain disease activity, and slows down progression of disability (18). The anti-inflammatory and tissue-protective mechanism of IFNβ likely involves anti-proliferative and pro-apoptotic effects, as well as induction of anti-inflammatory mediators such as IL-10, IL-1R antagonist and soluble TNF receptors and reduction of pro-inflammatory mediators such as IL-1, IL-6 and TNFα (19).

From the above, the question emerges why type I IFNs can be pathogenic in SLE but therapeutic in MS. It is tempting to speculate that despite mechanistic similarities, IFNα and IFNβ have distinct roles in immune regulation that confer these opposing effects. Comparison of the primary amino acid

sequences revealed that IFNα differs from IFNβ by approximately 70% (20). Receptor binding studies demonstrated that IFNα and IFNβ interact with their receptors in a different manner, suggesting that IFNα and IFNβ activate the IFNAR1/IFNAR2-mediated signal transduction pathway in a slightly different way (21-23). Accordingly, *in vitro* studies revealed that IFNβ appeared to be more potent at inhibiting cell proliferation and inducing apoptosis than IFNα (24). However, differences in the downstream gene activation program of IFNα- and IFNβ-induced IFN signatures *in vivo* are yet unknown.

In the present study, we used transcript profiling to compare the IFN signature gene components regulated by IFNα in SLE patients to those of IFNβ-treated MS patients. Moreover, we exploited our findings to delineate the nature of the type I IFN signature in IIM, RA and IFNβ-naïve MS patients.

Methods

Patient recruitment

SLE patients (n=47) and RA patients (n=76) were recruited at the Amsterdam Rheumatology and immunology Center, Amsterdam, The Netherlands. MS patients were recruited from a prospective European multicenter study that was previously described(25). For the untreated MS cohort, blood samples collected before start of IFNβ therapy were used (n=160); for the IFNβ-treated MS cohort, we used blood samples drawn after 3 months of IFNβ therapy (n=71). IIM patients (n=78) were recruited at the Rheumatology Unit at Karolinska University Hospital, Stockholm, Sweden or at the Institute of Rheumatology, Prague, Czech Republic, and fulfilled the diagnostic criteria for definite or probable polymyositis (n=32), dermatomyositis (n=40) or sporadic inclusion body myositis (n=5). Healthy controls (HC, n=54) were recruited at the VU University medical center, Amsterdam. All donors gave their informed consent. Demographic data, clinical information and medication use of the patients at the time of blood sampling are shown in **Table 1**.

Table 1 Patient characteristics for the complete cohorts ("All") or the IFN^{high} selection

		SLE	MS-IFNβ[2]	Untreated MS	IIM	RA	Healthy Controls
Total amount	All	47	71	160	78	76	54
	IFN^{high}	30	63	12	26	10	
Age in years, mean (SD)	All	44 (14)	34 (8)	36 (10)	56 (14)	54 (13)	35 (10)
	IFN^{high}	42 (13)	35 (8)	34 (9)	55 (17)	52 (16)	
Female, %	All	85	73	67	62	79	53
	IFN^{high}	93	72	83	69	89	
Disease activity, mean (SD)[1]	All	4 (5)	n.a	n.a.	n.a.	4.8 (1.4)[3]	n.a.
	IFN^{high}	5 (5)				5.3 (1.6)[4]	
Current prednisolone use, %	All	50	n.a.	n.a.	70	17	n.a.
	IFN^{high}	57	n.a.	n.a.	60	22	
Current use of other immunomodulatory drugs, %	All	63	n.a.	n.a.	60	24	n.a.
	IFN^{high}	67	n.a.	n.a.	56	33	

[1]Disease activity scores: SLE, SLEDAI; RA, DAS28. [2]Patients that were IFN^{high} before start of therapy were not included in the IFN^{high} selection of this cohort [3]data missing from 5 patients [4]data missing from 1 patient. IFN^{high}, presence of a type I IFN signature, see Figure 1; SD, standard deviation; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; DAS28, Disease Activity Score in 28 joints; n.a. not applicable.

Blood sampling and RNA isolation

From the donors in the SLE, IIM, RA and HC cohorts, 2.5 ml blood was drawn in PAXgene tubes (PreAnalytix GmbH, Hombrechtikon, Switzerland) and stored at -20°C. After overnight thawing at room temperature total RNA was isolated according to the manufacturer's instructions (PAXgene Blood RNA kit). Total RNA concentration was measured using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). From the donors in the untreated MS cohort and the IFNβ-treated MS cohorts, blood was collected in a Tempus tube (Applied Biosystems, Foster City, California, USA), and processed as described before [25].

Reverse transcription and pre-amplification of cDNA

RNA (0.5 µg) was reverse-transcribed into cDNA using a Revertaid H-minus cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany). A single aliquot of each cDNA sample, equivalent to 12.5 ng RNA, was first subjected to 14 cycles of Specific Target Amplification using a 0.2X mixture of all Taqman Gene Expression assays in combination with the Taqman PreAmp Master Mix (Applied Biosystems, Foster City, California, USA). Following pre-amplification, the samples were diluted 1:5 (v/v) in TE buffer, pH 8.0.

Multiplex Real-Time PCR

Custom-designed TaqMan®assays for each gene were supplied by Applied Biosystems. qPCR analysis was performed at ServiceXS (ServiceXS B.V., Leiden, The Netherlands) using the 96.96 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA, U.S.A), according to the manufacturer's instructions. Thermal cycling and real-time imaging of the BioMark array was done on the BioMark instrument, and C_T values were extracted using the BioMark Real-Time PCR analysis software. Relative quantities were calculated using the standard curve method, using GAPDH as a housekeeping gene. Expression levels were $\log_2$-transformed.

Calculation of the type I IFN score and selection for initial analyses

Based on whole genome expression data available in literature, 23 IRGs (see Supplementary **Table 1**) were selected that are reflective of a communal type I IFN signature between autoimmune diseases. All 23 genes are described to be upregulated compared to healthy controls in one or more of the autoimmune diseases SLE, MS, IIM and/or RA (9;13;26-30). To control for inter-experimental variation, expression levels of each gene were calculated relative to its median expression in healthy controls. Since all IRGs were highly correlative (Pearson $r>0.7$ for 90% of the combinations, $p<0.0005$), we calculated an IFN score by averaging the expression levels of all IRGs per sample.

Presence of a type I IFN signature (referred to as IFN^{high}) was defined as an IFN score above mean + 2*SD in HCs (1.3). To exclude the possibility that observed qualitative differences are actually due to quantitative differences, we selected IFN^{high} patients within a comparable range of IFN score, between 2.5 and 4, for initial analysis (**Figure 1**). The remaining IFN^{high} patients were used as an additional cohort to verify our initial findings.

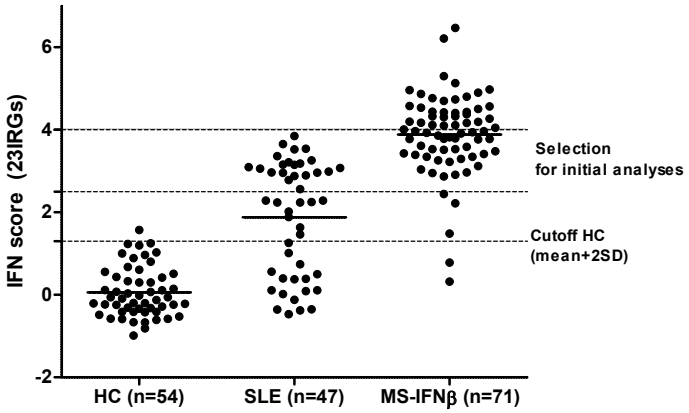

Figure 1 IFN score in SLE patients and MS patients who received 3 months of IFNβ treatment. Average expression levels of 23 IRGs show a comparable range for the majority of SLE and IFNβ-treated MS patients. SLE and IFNβ-treated MS patients with an IFN score between 2.5 and 4, as shown in the grey area, were selected for the initial comparison of the composition of the IFN signature. IFN^{high} patients with an IFN score above 4 or below 2.5 were used as an additional cohort.

Statistical analysis

Cluster analysis was used for categorization of IRGs with respect to their relative expression between diseases (31). TreeView was used to visualize the clustering of genes (Eisen Lab, Berkeley, California, USA). Comparison of IRG expression between SLE and IFNβ-treated MS patients was performed using unpaired t-tests, with multiple testing correction using the Benjamini-Hochberg method. Comparison of IFN scores between SLE and MS-IFNβ was performed using unpaired t-tests and comparison of cluster-specific IFN scores within patients was performed using paired t-tests. P values <0.05 were considered significant.

Results

Differential expression of IRGs in SLE versus IFNβ-treated MS patients

In order to explore *in vivo* differences in the composition of type I IFN signatures in autoimmune diseases, we studied IRG expression profiles of a prototype IFNα-driven disease, i.e. SLE, and those of MS patients who were treated with IFNβ for 3 months. As described above, only patients with an IFN signature (referred to as IFN^{high}) were included for further analysis. To ensure that the observed IFN signature was specifically induced by the IFNβ treatment, MS patients with an IFN signature before start of IFNβ treatment were excluded from analysis. For initial analysis, we used data from patients with comparable levels of IFN score, between 2.5 and 4, as described above.

To compare the IFN signature gene components regulated by IFNα in SLE to those of IFNβ-treated MS patients, unsupervised cluster analysis was performed (**Figure 2A**). Strikingly, the analysis revealed a perfect separation of SLE patients and IFNβ-treated MS patients based on two IRG clusters. From the upper cluster, 5 out of 7 IRGs (GC-A) were significantly upregulated

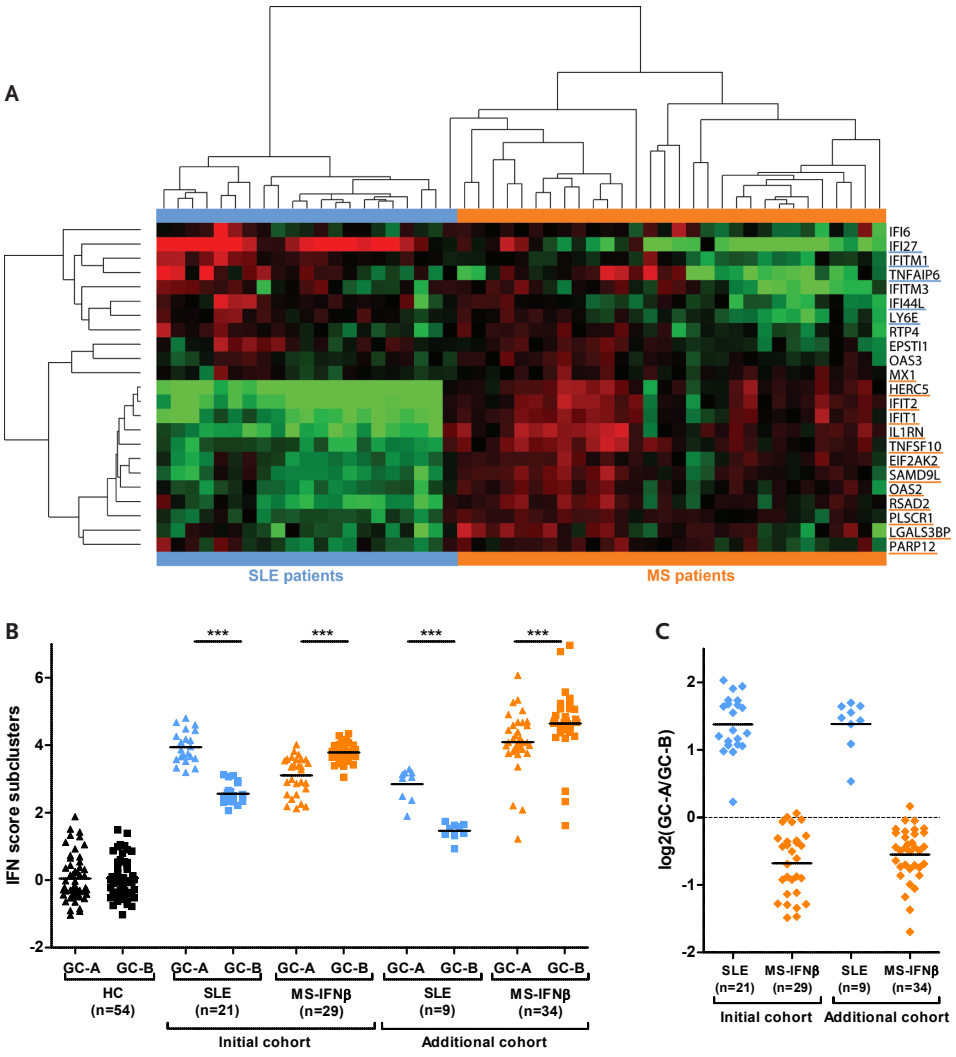

Figure 2 Comparison of gene clusters in SLE and IFNβ-treated MS patients. A) Unsupervised cluster analysis of IFN^{high} SLE and IFNβ-treated MS patients. Patient groups were fully separated based on their expression profiles of 23 IRGs. Separation is based on differential expression of two major gene clusters. Significantly different genes comprising GC-A (blue) and GC-B (orange) are underlined; B) GC-A and GC-B scores were compared in SLE and MS patients in the initial and additional cohort. In both cohorts, GC-A score is higher than GC-B score in SLE patients whereas the opposite is true for IFNβ-treated MS patients. C) The log-ratio of GC-A and GC-B scores was compared in SLE and MS patients of the initial and additional cohort. In all SLE patients, the ratio is above zero, indicating that GC-A > GC-B. In virtually all IFNβ-treated MS patients, GC-A/GC-B ratio is below zero, indicating GC-B > GC-A

in SLE patients compared to MS-IFNβ, whereas 13 out of 16 genes (GC-B) from the lower cluster were significantly upregulated in the IFNβ-treated MS patients compared to SLE (**Figure 2A, Supplementary Table 2**). GC-A and a GC-B scores were calculated by averaging expression values of these 5 and 13 genes, respectively. As shown in **Figure 2B**, the GC-A score was significantly higher than GC-B in SLE ($p<0.001$)whereas the GC-B score was significantly higher than GC-A in

IFNβ-treated MS ($p<0.001$). Analysis of IFN^{high} patients with IFN scores lower than 2.5 or higher than 4 confirmed these findings (additional cohort, **Figure 2C**). To gain insight into the relative importance of each gene cluster per patient, the GC-A/GC-B ratio was calculated. Since this ratio is based on $\log_2$-values, a ratio above zero means higher GC-A score compared to GC-B, whereas a ratio below zero means that the GC-B score is higher than the GC-A score. Comparison of these GC-A/GC-B log-ratios revealed that SLE and MS patients from both cohorts could be completely separated from each other based on these ratios (**Figure 2C**).

Validation in public microarray datasets

In order to validate our observations, publicly available microarray data was downloaded from the Gene Expression Omnibus database of the National Center for Biotechnology Information (32). Dataset GSE17755 contains gene expression data of peripheral blood cells from 25 healthy individuals and 22 SLE patients (33). Datasets GSE41846 and GSE41848 consist of combined gene expression data of peripheral blood cells from 38 healthy controls and 78 IFNβ-treated MS patients (34). Expression data for the 23 IRGs were extracted from these datasets, except for SAMD9L as it was not available in all sets. Patients with an IFN signature were selected based on the HC cut-off, as described above, and GC-A/GC-B log-ratios were determined. As shown in **Figure 3**, these data confirmed our findings: SLE patients displayed a dominant GC-A score, whereas GC-B dominance was apparent for the majority (78%) of IFNβ-treated MS patients. A small proportion of IFNβ-treated MS patients showed some GC-A dominance, which might be explained by the fact that we could not exclude patients with an IFN signature before start of therapy, as the dataset did not contain paired data before and during IFNβ treatment for all patients. Altogether, these data confirm the presence of IFNα- and IFNβ-specific signatures and the utility of the GC-A/GC-B log-ratio to distinguish between those signatures.

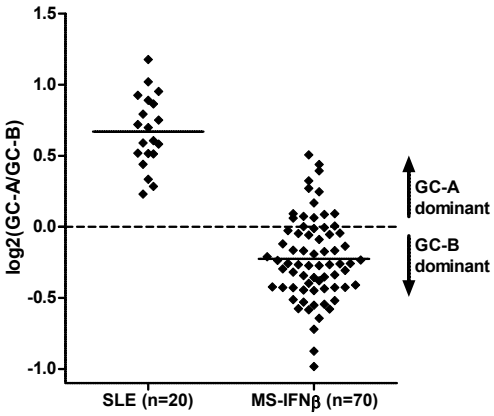

Figure 3 Validation of our findings in publicly available microarray data. The ratio between GC-A and GC-B was calculated for independent validation cohorts of 20 IFN^{high} SLE patients and 70 IFN^{high} IFNβ-treated MS patients. This confirms our earlier findings of GC-A>GC-B in SLE and GC-B>GC-A in IFNβ-treated MS

Expression of gene clusters in other autoimmune diseases

The above results indicate that IFNα and IFNβ-driven type I IFN signatures can be distinguished based on the GC-A/GC-B log-ratio. Thereto, we determined the GC-A/GC-B log-ratio in patients with idiopathic inflammatory myopathies (IIM), rheumatoid arthritis (RA) patients and IFNβ-naïve MS patients, autoimmune diseases with type I IFN signatures of yet unknown origin. Again, only IFN^{high} patients were selected.

As shown in **Figure 4A**, IIM showed GC-A dominance as reflected by positive GC-A/GC-B log-ratios, indicating predominant IFNα activity similar to SLE. The GC-A/GC-B log-ratio in RA patients was lower and approached zero in part of the patients, indicating contribution of the

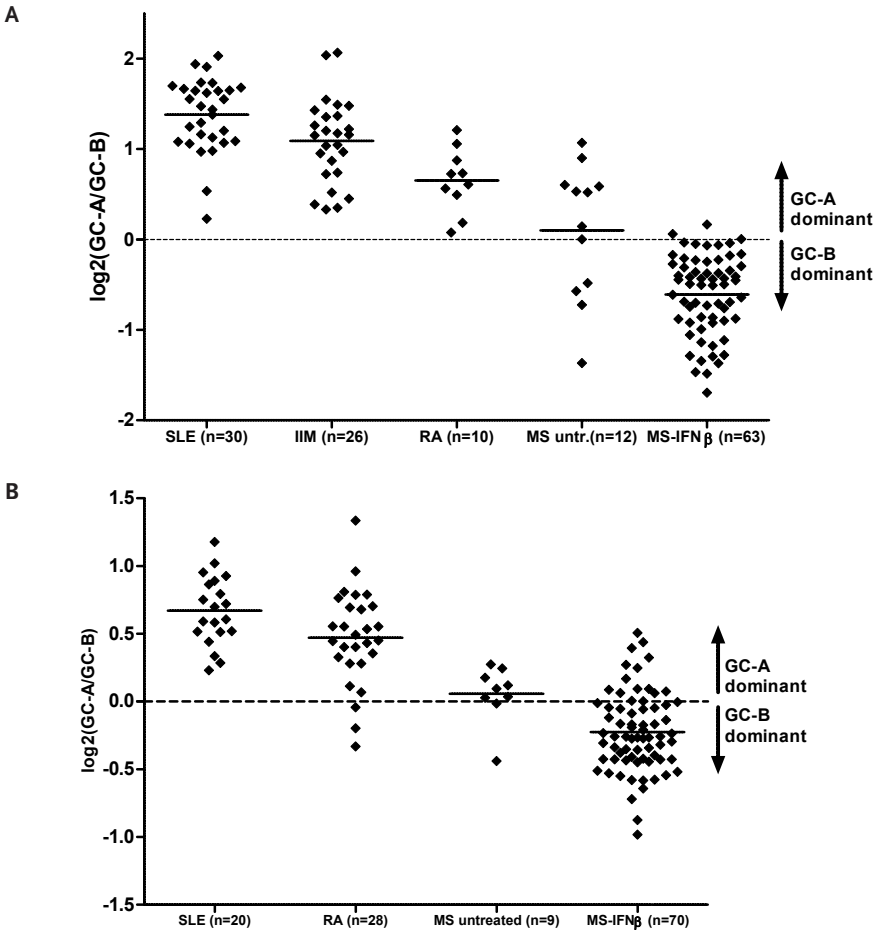

Figure 4 Comparison of gene clusters in autoimmune diseases. A) Log_2(GC-A/GC-B) was compared between SLE, untreated MS, IIM and RA patients. The GC-A/GC-B log-ratios are comparable between SLE and IIM. RA patients display less distinctive log-ratios of GC-A and GC-B. Untreated MS patients are characterized by either GC-A or GC-B dominance. B) Confirmation of these findings using publicly available microarray data.

GC-B cluster as well. Remarkably, untreated MS patients appeared most heterogeneous; approximately half of the patients were characterized by GC-A dominance, whereas the other patients displayed GC-B dominance or a log-ratio close to zero.
These findings were validated for IFN^{high} RA and untreated MS patients, as the microarray datasets used for validation of our findings in SLE and IFNβ-treated MS patients also contained gene expression data of RA patients (GSE17755, n=112) and untreated MS patients (GSE41846 and GSE41848, n=62) (**Figure 4B**) (33;34).

Transcriptional regulation of IRG gene clusters

To explore functional differences between GC-A and GC-B genes, a transcription factor binding site (TFBS) analysis was performed on these gene clusters using the interferome database and rVISTA (35;36). Interestingly, output from the Interferome database showed that prototypical IFN-response elements ISRE, ICSBP/IRF8 and IRF7 are mainly present in the GC-B genes and not in the genes of GC-A (**Figure 5**). This was supported by statistical analysis for TFBS enrichment using rVista, which showed significant enrichment in GC-B of both IRF8-binding sites and ISRE (within a 100bp upstream regulatory region, p<0.0001 and p=0.02, respectively). No enrichment of IFN-related TFBS was found in the GC-A geneset (data not shown). This indicates differential transcriptional regulation of the GC-A and GC-B genes, further supporting different upstream activity.

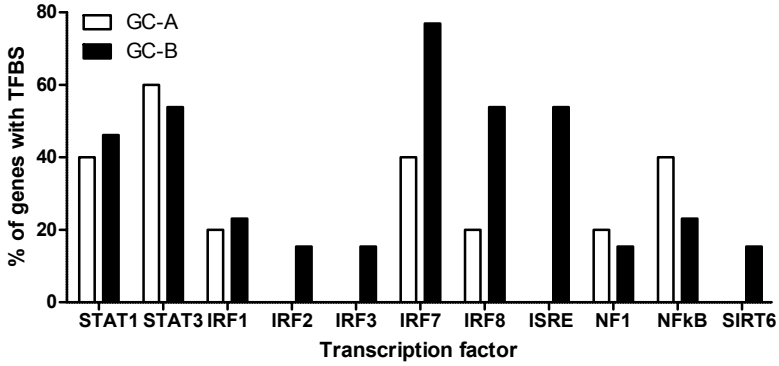

Figure 5 Transcription factor binding site analysis using the Interferome database. Represents the presence of transcription factor binding elements 1500bp upstream from the transcription start site. IRF7, IRF8 and ISRE are mainly present in genes from GC-B and not in genes from GC-A

Discussion

Presence of a type I IFN signature is often discussed as a similarity among autoimmune diseases. In the present study we provide evidence that type I IFN signatures in autoimmune diseases appear less uniform than generally assumed.

IRG expression patterns were different between SLE and IFNβ-treated MS, two autoimmune diseases in which IFN activity has opposing effects on immune pathology and regulation, believed

to be a consequence of differential effects of IFNα and IFNβ. Log-ratios of the differentially expressed type I IFN gene clusters, designated GC-A (SLE-IFNα-related) and GC-B (MS-IFNβ-related), revealed excellent separation of SLE and IFNβ-treated MS patients. Moreover, use of the GC-A/GC-B log-ratios in RA, IIM and IFNβ-naïve MS patients provided insight into the origin of the type I IFN signatures in these diseases.

The GC-A/GC-B log-ratios revealed that IIM is predominantly characterized by a GC-A, hence SLE-like, IFN signature. This fits with the many similarities between SLE and IIM that have been reported previously, including those related to the IFN pathway (Vosslamber et al., 2015, submitted) (37). With regard to RA, the GC-A/GC-B log-ratio was close to zero for some patients, indicating that both IFNα and IFNβ contribute to the IFN signature, as has been suggested before (38). Interestingly, GC-A/GC-B log-ratios differed among IFNβ-naïve MS patients, as half of the patients showed GC-A dominance, and others showed GC-B dominance or ratios close to zero. This could implicate different mechanisms underlying the type I IFN pathway activation in these patients. We previously showed that presence of a baseline IFN signature in MS patients is related to non-responsiveness to IFNβ treatment (27) and it is highly relevant to further investigate the role of GC-A and GC-B in this perspective, which is the objective of future studies. Altogether, these results suggest a differential role of type I IFNs in autoimmune diseases.

The considerable variance of type I IFNs in humans suggests that, although they bind to the same receptor, the effects they exert might be different. For example, it has been shown that IFNβ is more potent than IFNα in inhibiting proliferation, inducing apoptosis and cell differentiation (39;40). Differences were partly explained by the different affinities of type I IFNs for their receptors, resulting in different receptor trafficking, phosphorylation and signaling kinetics (21;41). More recently, it has been described that IFNβ can uniquely ligate to IFNAR1, independently of IFNAR2 (42). Overall, IFNβ appears more potent in activating signal transduction than IFNα, as demonstrated by a more stable receptor complex formation (43), a lower EC_{50} for ISGF3 phosphorylation (44) and induction of a larger amount of genes than IFNα, especially at long incubation times of 16-36h (45). Notably, these long incubation times conform to the chronic IFNα exposure in SLE and 3 months of IFNβ therapy in MS. Interestingly, Moraga et al. hypothesized that the short-term complex formation of IFNα with its receptors might cause a constant low level of apoptosis, whereas the long-term complex formation of IFNβ with its receptors could more potently induce high levels of apoptosis. Since SLE pathology is characterized by impaired clearance of apoptotic cells, resulting in immune complex formation and consequent IFNα induction, the low levels of apoptosis as mediated by IFNα could be key to persistence of a vicious pro-inflammatory circle.(46) In MS, however, apoptosis of autoreactive T cells is considered to be one of the anti-inflammatory actions of IFNβ therapy (47).

The implication of IFNα-and IFNβ-specific signatures is supported by the experiments of Der et al., who performed an in-vitro experiment in which the fibrosarcoma cell line HT1080 was stimulated with either IFNα or IFNβ, followed by gene expression measurements using oligonucleotide arrays for ±6800 genes (48). From these experiments, 7 genes overlap with our gene clusters, one from the GC-A cluster and six from the GC-B cluster. IFITM1, a GC-A gene, showed a slightly higher expression in IFNα-stimulated cells compared to IFNβ, whereas the GC-B genes

EIF2AK2, IFIT1, IFIT2, MX1, OAS2 and PLSCR1 were all induced more by IFNβ than by IFNα (1.2-fold to 7-fold higher induction) (48). Despite the small overlap of genes, the consistency of these results is striking.

It has been suggested that the type I IFN response differs among immune cell types (49), implying that the observed differences between SLE and MS could be partly due to differences in immune cell composition. However, the agreement of our data with those of Der et al. suggests that the observed differences are due to consistent differential signaling in all cells rather than large differences in immune cell compositions or IFNAR expression. However, for replication and complete definition of IFN subtype specific response programs whole genome expression studies are required.

Analysis of transcriptional regulation of GC-A or GC-B genes showed enrichment for IRF8/ICSBP binding sites and ISRE in the GC-B cluster. Remarkably, none of the IRGs from the GC-A cluster contained an ISRE, the response element that binds the ISGF3 complex downstream of canonical type I IFN signaling. As they did contain binding sites for STAT1 and/or STAT2, they are probably induced via STAT1-STAT1 monomers or STAT1-STAT2 heterodimers, which both IFNα and IFNβ are able to activate (50;51). The observation that these genes are increased in SLE compared to IFNβ-treated MS might be explained by the indication that IFNβ, in contrast to IFNα, might more potently activate a broader range of signaling proteins, including ISGF3 and IRF8, resulting in relatively less activation of the GC-A genes by IFNβ.

Expression of IRF8 is restricted to immune cells and it has the ability to act as a repressor or activator of the IFN response, depending on its interaction partner. A study by Meraro et al. reported that the IRF1-mediated induction of the IFN response gene ISG15 was inhibited in presence of IRF8, whereas interaction of IRF8 and PU.1 synergistically enhanced ISG15 induction (52). This suggests an immunomodulatory role for IRF8, which might be key to the different effects of IFNα and IFNβ on the immune system.

Conclusively, this study demonstrated that the IFN signatures display distinct differences between autoimmune diseases. Considering the pro-inflammatory nature of IFNα in SLE and the anti-inflammatory role of IFNβ in MS, specification of the type I IFN response in autoimmune diseases might give new insights into its role in pathology and/or its therapeutic potential.

Acknowledgements

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Supplementary data

Supplementary Table 1 List of 23 type I IFN response genes that were measured

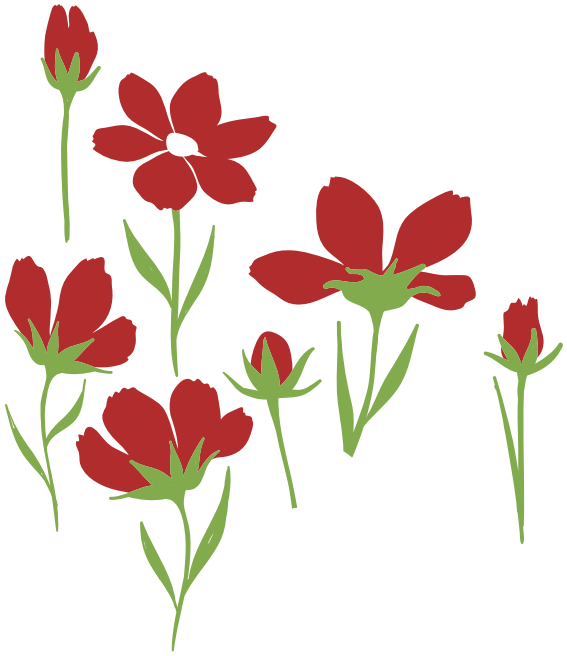
Gene Symbol	Gene Name
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2
EPSTI1	epithelial stromal interaction 1
HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5
IFI27	interferon, alpha-inducible protein 27
IFI44L	interferon-induced protein 44-like
IFI6	interferon, alpha-inducible protein 6
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
IFIT2	interferon-induced protein with tetratricopeptide repeats 2
IFITM1	interferon induced transmembrane protein 1
IFITM3	interferon induced transmembrane protein 3
IL1RN	interleukin 1 receptor antagonist
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein
LY6E	lymphocyte antigen 6 complex, locus E
MX1	MX dynamin-like GTPase 1
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa
OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa
PARP12	poly (ADP-ribose) polymerase family, member 12
PLSCR1	phospholipid scramblase 1
RSAD2	radical S-adenosyl methionine domain containing 2
RTP4	receptor (chemosensory) transporter protein 4
SAMD9L	sterile alpha motif domain containing 9-like
TNFAIP6	tumor necrosis factor, alpha-induced protein 6
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10

Supplementary Table 2 Genes differentially expressed between SLE and IFNβ-treated MS patients

Gene name	Fold difference	Adjusted P value
Upregulated in SLE patients (GC-A)		
IFI27	7.56	<0.001
IFI44L	1.39	0.006
IFITM1	1.69	<0.001
LY6E	1.32	0.006
TNFAIP6	1.73	0.003
Upregulated in IFNβ-treated MS patients (GC-B)		
EIF2AK2	1.75	<0.001
HERC5	4.63	<0.001
IFIT1	3.11	<0.001
IFIT2	3.86	<0.001
IL1RN	3.11	<0.001
LGALS3BP	1.68	<0.001
MX1	1.14	0.044
OAS2	1.77	<0.001
PARP12	1.27	0.009
PLSCR1	1.59	<0.001
RSAD2	1.93	<0.001
SAMD9L	1.92	<0.001
TNFSF10	2.13	<0.001

Chapter 4

General discussion



In the past years, presence of a type I IFN signature has been described in several autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), idiopathic inflammatory myopathies (IIM), multiple sclerosis (MS), systemic sclerosis, Sjögren's syndrome and type I diabetes (1-3). Hence, the IFN signature has become a more general phenomenon, and by in-depth understanding and implementation of this signature, we could advance our understanding and efficient treatment of such complex diseases. In this thesis, possibilities for clinical implementation of the IFN signature in RA were assessed, and studies focusing on the origin of the type I IFN response in rheumatic diseases were performed. These findings have led to new insights regarding the IFN signature in RA, but also raised questions and provided perspectives for future research.

Clinical utility of the type I IFN signature in rheumatoid arthritis

The IFN signature does not appear to be a disease activity parameter in RA

In **Chapter 2.1**, we demonstrated that the IFN signature in established RA patients was not associated with clinical parameters. Hence, the signature does not appear to reflect disease activity or severity per se. However, the known associations of the IFN signature with the risk of arthritis development as well as (non-)response to therapy in RA indicate that the IFN signature represents at least a subgroup of RA patients, as already pointed out by van der Pouw Kraan et al.(4). Even though there are no differences in symptoms, disease activity, laboratory measurements and autoantibodies between patients with and without this signature, there could be differences in the activation of pathophysiological pathways, and thereby in the activation and actions of cell types. This could eventually result in the same clinical symptoms that constitute RA, but might have crucial consequences for the response to therapy. In particular this holds true for biologics, as these are modulators of specific signaling pathways. The high incidence of approximately 30-40% non-response to every biologic used for RA (5;6) supports this hypothesis.

Combining predictive parameters improves the prediction performance

The above described heterogeneity of RA underlines the need for personalized medicine and, accordingly, the need for predictors of (non-)response. Fortunately, many efforts have been made to this end. A remarkable example is anti-TNF therapy, the first biologic therapy that became available for RA. Several studies have been published which describe markers of response prediction, including clinical parameters (7;8), genetic markers (9;10), protein markers (11;12) and transcriptomic markers (13-15). However, none of the non-clinical markers has been adequately validated. Initiatives to investigate the combined predictive power of these different markers remain scarce, while this hypothetically could be the solution to the apparent lack of validation (16;17).

In this thesis, we investigated the prediction of non-response to rituximab (RTX). Similar to anti-TNF therapy, multiple predictors were described for rituximab, including the IFN response gene (IRG) set, reflective of the IFN signature, by Raterman et al.(18), but the combined performance of these predictors was never thoroughly assessed (19-22). **Chapters 2.2** and **2.3** elaborated on the IRG predictor by studying the influence of prednisone treatment (**Chapter 2.2**) and investigating the combinatory power with other predictors (**Chapter 2.3**).

We demonstrated that the combination of disease activity, DMARD use and the IRG set provided excellent predictive performance, highly exceeding the performance of the individual parameters. This shows that the mechanisms by which these parameters contribute to (non-)response to rituximab are largely independent of each other.

The effect of prednisone use on IRG-based response prediction

In **Chapter 2.2**, prednisone use appeared to be a hampering factor in the IRG-based prediction of non-response to rituximab, which was also confirmed in **Chapter 2.3**. Prednisone, as all glucocorticoids, is a known suppressor of type I IFN signaling. The mechanisms of suppression are thought to involve competition of the glucocorticoid signaling cofactor GRIP1/NCOA2 with interferon regulatory factor (IRF)3 and IRF9, resulting in interference between glucocorticoid signaling and toll like receptor (TLR) signaling and IFNα/β receptor (IFNAR) signaling, respectively (23;24), and the glucocorticoid-mediated induction of SOCS1, an inhibitor of JAK-STAT signaling, including type I IFN signaling (25).

Our data suggest that optimal clinical utility of the IFN signature might be achievable in patients who are not using prednisone. Unfortunately, complete tapering of prednisone treatment prior to performing the IFN-based prediction test appears not feasible nor ethically desirable. We observed that the prednisone-related downregulation of the IFN signature was dependent on prednisone dose and also appeared dependent on the duration of treatment, i.e. cumulative dose, suggesting that the IFN-based prediction could still be applicable in prednisone-using patients under certain conditions. Future prospective studies should focus on the effect of actual and cumulative dosing of prednisone on the IFN signature, in order to strictly define the window within which the IFN-based prediction test could be successfully applied. Moreover, in **Chapter 2.3**, another prediction model was proposed, which was selectively developed in patients who had been on prednisone for at least one month. This model did not contain the IRG set, but consisted of DAS28 and autoantibody positivity and displayed an acceptable performance. Also, the other predictive variables besides the IFN signature did not appear to be influenced by prednisone use. Altogether, this implies that the optimal multivariate model would exist of disease activity and autoantibody positivity, and potentially the IFN signature, including a weigh factor calculated in relation to prednisone use, dose and duration of treatment.

The data from **Chapter 2.1**, which also showed suppression of the type I IFN signature in RA by hydroxychloroquine (HCQ) and sulphasalazine (SSZ), but not by MTX, suggest that these are also treatments that might interfere with the IFN-based response prediction of RTX. Although these regimens are used less frequently than prednisone, it might be valuable to study their effects on this prediction.

Clinical utility of the IFN signature for prediction of other biologics

Whereas this thesis is focused on the use of the IFN signature in RA in relation to rituximab response prediction, the IFN signature has also been described to play a role in response to other treatments . Although not reproduced yet, it has been demonstrated that presence of the IFN signature was also related to a good response to tocilizumab (26) and, when selectively determined in circulating neutrophils from RA patients, the IFN signature appeared to be related to a

good response to anti-TNF therapy as well (27). The clinical relevance of these findings remains to be determined, but it might be worthwhile to further study this in relation to both prednisone use and in combination with other clinical parameters.

Clinical utility of the IFN signature for early diagnosis

Besides the association of the IFN signature and the response to biologic treatment, presence of the IFN signature in patients at-risk for RA was also shown to be related to a higher risk of arthritis development (28;29). Determination of the IFN signature may therefore enable selection of high risk individuals that may benefit from early, or even preventive treatment.

Whereas the interference of prednisone treatment with IFN signaling is considered an impeding factor when the IFN signature is used to predict the non-response to treatment, conversely it might be exploited to delay or prevent disease onset in preclinical RA. In a previously described trial using the glucocorticoid dexamethasone in seropositive arthralgia, a preventive effect on arthritis development was not observed (30) but this was never studied in relation to patients with an IFN signature. Although current specificity of the IFN signature in preclinical RA might be too low to ethically allow pre-treatment with an immunosuppressant such as prednisone while there are no signs of inflammation yet (29), specificity might be improved by combination of the IFN marker with other predictive clinical parameters, such as family history, symptom duration and autoantibody positivity (31), and imaging data (32;33). If the specificity of the prediction of arthritis development could be increased, early immunosuppressive treatment might be feasible without over-treating too many misclassified arthralgia patients who do not develop arthritis.

Molecular characterization of the type I IFN signature in rheumatic diseases

The second part of this thesis was focused on molecular characterization of the type I IFN signature. The three chapters of this part approached this issue from different perspectives, ranging from up- to downstream IFN signaling; whereas in **Chapter 3.1** the upstream activation of the IFN signaling pathway was studied, **Chapter 3.2** was focused on the identification of the cell type(s) in which this signaling would take place, and in **Chapter 3.3** the composition of the IFN response gene program itself was investigated.

The upstream activator of the type I IFN response in RA

In **Chapter 3.1**, we studied whether serum from RA patients was able to induce an IFN response in healthy peripheral blood mononuclear cells (PBMCs). This type of bioassay has been successfully applied in the field of SLE, and has contributed greatly to understanding the mechanism behind the type I IFN response in this disease. In our study, we were able to demonstrate the presence of an IRG inducing factor in serum from a part of the RA patients. Although we did not yet succeed to exactly identify the factor that was responsible for the IRG induction, it was established that the process by which the IFN response took place was not equal to that of SLE. Our data indicate that the factor responsible for the IRG induction by RA serum functions via an indirect signaling route, i.e. it probably induces type I IFN protein which subsequently activates the IRG induction. Potentially, the serum levels of the type I IFN protein itself remain too low to detect, which would explain why literature on the detection of type I IFN protein in rheumatoid arthritis serum has been yet inconclusive (34-37).

Typical pathways of type I IFN induction are the activation of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), in particular TLR3, TLR4, TLR7 and TLR9, and cytosolic RNA helicases like RIG-I and MDA5 (38). However, since there has not been any conclusive evidence for presence of an active infection in RA patients, activation of these receptors probably does not occur via the conventional pathogen-derived ligands such as pathogen-derived nucleic acids, lipids, lipoproteins or peptidoglycans (38). Instead, endogenous TLR ligands, which existence is increasingly recognized, may seem suitable candidates for inducing the type I IFN response in RA. In particular endogenous ligands for TLR4 have been described, such as HMGB1, HSP70, Tenascin-C and S100A8/S100A9. Both TLR4 and these endogenous ligands are shown to be elevated in RA and could be potential initiators of the observed IRG induction (39-42).

Recent literature also suggests a role for certain cytokines in the induction of type I IFNs. Of particular interest for RA is the described connection between TNFα and type I IFN. It has been described that TNFα is able to induce IFNβ via an IRF1-dependent pathway, leading to IRG expression within 8h and in a CHX-dependent manner (43). The patients in our cohort have been off anti-TNF therapy for at least 4 weeks, hence TNFα levels might have been increased since then. On the other hand, there also have been reports on an increase in IRG levels upon anti-TNF treatment in RA, though not in all patients but selectively in non-responders (44;45). This would contradict an IFN response-inducing effect by TNFα in some RA patients. Bienkowska and colleagues showed that the type I IFN signature in RA was decreased upon treatment with baminercept, an inhibitor of lymphotoxin-β receptor (LTβR) signaling, implying a potential role for LTβR-binding cytokines as well (46). Future studies could include performing the bioassay in the presence and absence of inhibitors of the TLR-, TNFα-, LTβ- and LIGHT-mediated signaling in order to identify their contribution to the IRG induction in RA.

Other contributors to the IFN signature

It is important to consider that the IFN inducing capacity of a serum component is not necessarily the only causative factor behind an IFN signature. Although presence of an agonist is most probably required for the initial pathway activation, the signature might be enhanced or maintained by a combination of increased sensitivity for this agonist and/or disturbances in the downstream signaling pathway, such as increased activity of signaling components and/or impaired functioning of negative feedback routes (**Figure 1**).

Potential signaling disturbances in RA, or maybe just inter-individual variation in signaling potency, might have evolved from genetic variation among patients. Variation in the genes encoding for STAT4 and IRF5, both proteins involved in type I IFN signaling, have been described to be associated with an increased susceptibility to develop RA (47;48). Since some of the gene variants have shown to result in higher expression of the encoded proteins (49;50) and since these proteins have been shown to induce expression of components of the type I IFN signaling pathway (51), patients with SNPs in one or both genes could show a higher potency in IFN signaling than patients expressing the common variant, and therefore be "susceptible" to having an IFN signature. In fact, Delgado-Vega and colleagues described many more genetic variants that were associated with rheumatic diseases and encoded proteins involved in type I IFN signaling, which further emphasizes the potential complexity behind the IFN signature (52).

Furthermore, our data described in **Chapter 3.2** indicate that early arthritis patients, including RA patients, also display increased sensitivity to IFN signaling, particularly in the neutrophils (PMNs). Although all studied leukocytes, i.e. T cells, B cells, monocytes and PMNs, displayed elevated IRG induction in patients with a whole blood IFN signature, the contribution of the PMNs to this signature appeared to exceed those of the other cell fractions. In comparison with healthy control cells, PMNs displayed elevated expression of the type I IFN receptors, IFNAR1 and IFNAR2, which implies that the PMNs would be more receptive to circulating type I IFNs.

The identified connection between the IFN signature and the PMNs gives new insight into a potential role of the IFN signature in RA pathology. The role of the PMNs in RA is well-established, but the last few years attention has shifted more towards the role of adaptive immune cells in the disease. However, the PMNs are the most early and abundant cell type present in the RA joint (53;54), indicating that it could contribute to the first joint damage. It has been shown that type I IFNs are able to prolong the lifespan and functioning of neutrophils (55;56), which suggests that PMNs of patients with an IFN signature may be more active compared to patients without this signature. Of note, in **Chapter 2.1** we did not observe an association of the IFN signature with the presence of erosive disease as measured by conventional radiography. However, proper assessment of this association, and the relation with PMN activity, might require more sensitive techniques such as ultrasonography, CT or MRI in order to determine the number and severity of erosions.

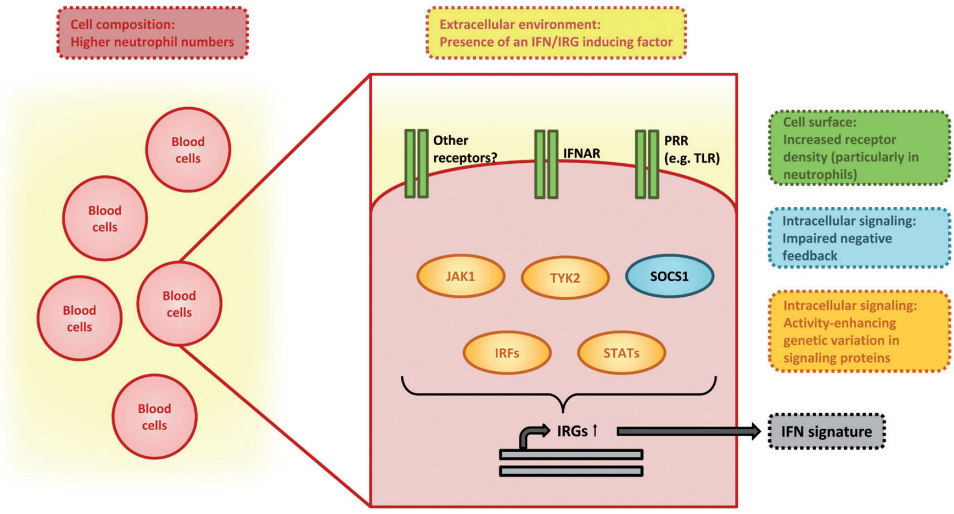


Figure 1 Schematic overview of possible contributors to the peripheral blood IFN signature in rheumatoid arthritis. IFNAR, IFN alpha/beta Receptor; PRR, pattern recognition receptor; JAK1, Janus Kinase 1; TYK2, Tyrosine Kinase 2; SOCS1, Suppressor of Cytokine Signaling 1; IRF, IFN Regulatory Factor; STAT, Signal Transducer and Activator of Transcription.

Dissecting the gene composition of IFN signatures

Besides the question on the origin of the type I IFN pathway activation, there is the issue of which type I IFN would be involved, which was addressed in **Chapter 3.3**. It has been suggested that these IFNs, despite binding to the same receptor, would exert different effects on the immune system. In general, IFNα is considered to be pro-inflammatory, whereas IFNβ is thought to have more anti-inflammatory effects. Prototypical examples fitting this theory are SLE, in which the pathological role of IFNα was well-established, and MS, in which IFNβ therapy has shown to be beneficial for many patients. Explanations for this difference are suggested to lie in the site of production (IFNα, systemic and IFNβ, local, reviewed in Crow 2010 (57)) and/or in the higher potency of IFNβ to induce apoptosis compared to IFNα (58;59). In Chapter 3.3, we defined IFN-subtype-dominant response programs, which suggested that there were differences in the activation of transcription factors by IFNα and IFNβ in peripheral blood leukocytes. We demonstrated that composition of the IFN signature in myositis patients resembled that of SLE patients, indicating that this signature is also IFNα-mediated. On the other hand, the IFN response in RA and MS patients appeared not as explicitly mediated by IFNα as observed in SLE and IIM patients, implying potential involvement of IFNβ as well, which has been suggested in literature as well (36;37).

The finding that the IFN signature in myositis is mediated by IFNα and thereby similar to SLE, corroborates with the earlier described observations that the IFN signature in both these diseases is associated with certain autoantibodies, often directed against nuclear components (60;61), indicating that IFN induction occurs via similar Fc Receptor and/or TLR signaling routes (62). With regard to RA, such an association has not been found, as also addressed in **Chapters 2.1** and **3.1**. Altogether, further studies are required to elucidate the exact involvement of IFNα and IFNβ in the IFN signature in RA.

It is also interesting to consider the possibility that the IRGs induction in RA patients might (partly) be mediated by pathways other than canonical type I IFN signaling. For example, Schoggins et al. demonstrated that certain viruses were able to induce IRGs in STAT1-negative cells, although it was not assessed whether this required intermediate production of type I IFNs (63). Alternatively, observations of TLR7-mediated direct STAT1 activation and subsequent IRG induction independent of type I IFN production has also been reported (64). Interestingly, the latest discovered type III IFN, IFNλ, has the ability to induce the same IRGs as type I IFNs. It would be interesting to study this in relation to the IFNα- and IFNβ-mediated response programs. Of particular interest with respect to RA, it was shown that the kinetics of IFNλ-mediated IRG induction was slower than that of IFNα, i.e. with an optimum after 8 hours of incubation instead of 4 hours like IFNα (65;66). This would fit with the IRG induction we observed in healthy PBMCs after 8h incubation with RA serum, as described in **Chapter 3.1**.

Conclusions and future perspectives

Altogether, we have progressed in both the clinical and the molecular characterization of the IFN signature in rheumatic diseases. However, complete unraveling of the IFN signature in RA, as well as in other autoimmune diseases, might actually require a systems biological approach,

which combines gene expression data, gene variation, epigenetics and preferably also protein expression and activity. With the rapid development of Next Generation Sequencing technologies this is becoming increasingly feasible (67-69), although analysis of such large data sets remains statistically challenging. This is particularly important considering the inter-individual variability among RA patients which will probably be reflected in such data.

Considering our and previously described findings regarding the IFN signature in relation to therapy response, one could speculate that patients with an IFN signature in the neutrophils might benefit from therapies that target the activity of neutrophil-derived cytokines, such as anti-TNF therapy (27) or tocilizumab therapy (26), whereas patients without an IFN signature might benefit from rituximab therapy instead (18) (**Figure 2**). It would be interesting to also study this in relation to other biologics, such as anakinra, an antagonist of the IL1 receptor which is expressed on neutrophils as well, and abatacept, an inhibitor of T cell co-stimulation. However, more studies on the exact role of the IFN signature in neutrophil-related RA pathology are required to support this hypothesis.

An important issue in relation to any pathogenic or pathologic role of the IFN signature in RA involves the long-term fluctuation of the signature. To this day, it is yet unknown whether the IFN signature that is detected in part of the arthralgia patients in fact correlates with the IFN signature that is detected in established RA patients. Insight into this longitudinal (in)stability could provide important information on its clinical applicability as a biomarker. That is, a stable signature would indicate that an IFN^{high} arthralgia patient who develops arthritis would not be recommended to use rituximab but rather might already benefit from anti-TNF and/or tocilizumab treatment (18;26;27;29)(see also **Figure 2**). Alternatively, when the IFN signature would be fluctuating and occasionally disappear, potentially this could be exploited to determine the optimal time point to start rituximab treatment. Furthermore, insight into the variation of the IFN signature between disease phases could also give information on whether and when it plays a potential anti-inflammatory or rather detrimental role in the disease onset and progress. Since the signature is highly variable between patients when determined in a cross-sectional manner (**4**), paired longitudinal measurements would be highly important though practically challenging.

In order to achieve further understanding of autoimmune diseases such as RA, it is essential to look beyond the IFN signature and define other specific disease subgroups. For example, the original paper on the IFN signature in RA already mentioned existence of a chemokine-related gene cluster and upregulation of a cluster of S100 family proteins, of which the latter was also reported in another gene expression study (4;70). Further exploration of these signatures could ultimately result in complete subclassification of RA and, hopefully, personalized treatment per subclass.

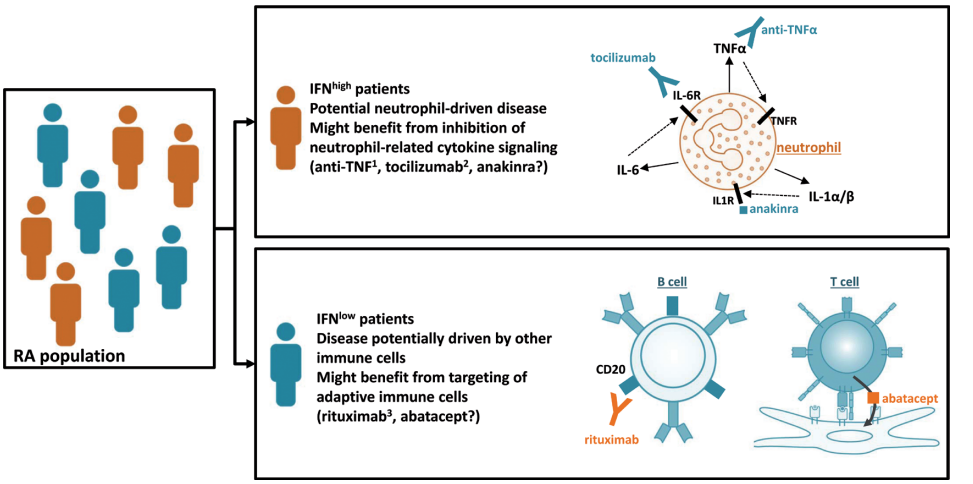


Figure 2 Hypothetical options for personalized biologic treatment based on the IFN signature in RA. Cell images adapted from Janeway's Immunobiology, 6th edition. 1Wright et al., 2015 (27); 2Sanayama et al.,2014 (26); 3Raterman et al., 2012 (18).

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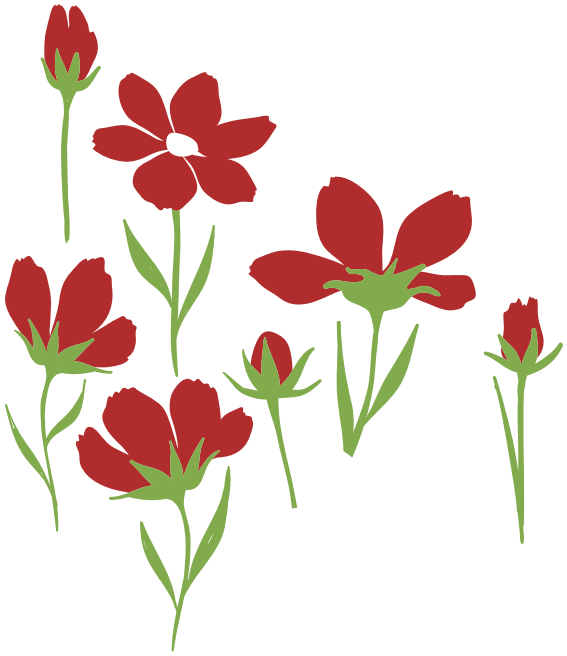
Chapter 5

Summary



Scope of the thesis

Rheumatoid arthritis (RA) is a challenging disease: due to its heterogeneous nature, there is large variability in disease severity, symptoms and response to therapy. Insight into the activated pathways in RA subgroups and/or predictors of therapy response is of vital importance to unravel its pathology and ultimately enable personalized treatment strategies for RA patients.
Type I interferon (IFN) activity has proven to play an important role in rheumatoid arthritis and other autoimmune diseases. Approximately 50% of RA patients displays a type I IFN signature, which has shown to be associated to a poor response to rituximab treatment. The research presented in this thesis was aimed to gain more insight into the clinical utility of the type I IFN signature in RA, as well as into the potential mechanisms behind the type I IFN signature in RA and other autoimmune diseases.

Clinical characterization of the type I IFN signature in rheumatoid arthritis

The first part of this thesis, which was described in **Chapter 2**, was focused on the potential clinical applicability of the IFN signature in RA.

One question that has been lingering since the IFN signature was described in RA for the first time in 2007, is whether it would be related to clinical parameters, as it appeared to represent a subgroup of patients. In **Chapter 2.1**, we investigated the association of the IFN signature with disease activity, inflammation parameters, erosive disease and antibody positivity, which did not reveal any significant associations. We did, however, observe downregulation of the signature upon treatment with hydroxychloroquine (HCQ), sulphasalazine (SSZ) and prednisone (PREDN).

Chapters 2.2 and **2.3** were aimed to optimize the previously described type I IFN-based prediction of the response to rituximab treatment in RA patients. In **Chapter 2.2**, we studied the effect of prednisone use on this prediction. Interference between glucocorticoids (GCs), such as prednisone, and type I IFN signaling has previously been demonstrated in vitro, and since the use and dose of oral GCs is highly variable among RA patients prior to the start of treatment with rituximab, we determined what the effect of GC use was on the type I IFN response gene (IRG) expression in relation to the clinical response to rituximab.

We demonstrated that expression of the 8 predictive IRGs was suppressed in RA patients who used prednisone at the time of blood collection. This suppression was dependent on prednisone use and most prominent in patients using ≥10mg/day prednisone. Consequently, the predictive performance of the IRG profile was lower in prednisone users ($PREDN^+$) compared to RA patients without prednisone use ($PREDN^-$); receiver operating characteristics (ROC) analysis in $PREDN^+$ patients resulted in an area under the curve (AUC) of 0.80, whereas an excellent AUC of 0.98 was reached for $PREDN^-$ patients. Using an IRG expression cutoff that would result in correct classification of all responders, 92% of the $PREDN^-$ patients were correctly classified, compared to only 63% of the $PREDN^+$ patients. In conclusion, these findings indicate that the IFN-signature-based rituximab response prediction could be optimized by stratification of prednisone use.

Chapter 2.3 was focused on the identification of clinical parameters that could predict the non-response to rituximab therapy in addition to the type I IFN response gene profile. This study resulted in a multi-parameter model containing 3 parameters –baseline disease activity score (DAS28), positivity for IgM-rheumatoid factor (IgM-RF) and antibodies against citrullinated proteins (ACPA) and the type I IFN response gene profile–, which displayed a good performance (AUC 0.84). The combination of predictive parameters greatly improved the prediction compared to prediction based on each parameter alone. However, upon validation in an independent cohort, we observed that prednisone again appeared a disturbing factor in the IFN-related response prediction. The validation cohort only contained PREDN^{+} patients whose dose had been stable for at least 4 weeks, whereas this was not the case in the test cohort, implying that these patients might have reached higher cumulative doses of prednisone than the patients in the test cohort. We hypothesize that the effect on the IFN signature would depend on cumulative dosing, i.e. the dose combined with the duration of treatment.

Altogether, presence of the IFN signature does not seem to reflect a certain disease subtype or state based on clinical parameters. However, it is related to a poor response to rituximab, which might be clinically applicable to predict non-responders prior to start of treatment. In order to achieve this, combination with other predictive (clinical) parameters and exact definition of the suppression by prednisone are highly important.

Molecular characterization of the type I IFN signature in rheumatic diseases

Besides clinical applicability of the type I IFN signature in rheumatoid arthritis, the exact mechanism behind this signature is largely unknown. More insight into the mechanism of type I IFN activity in RA could provide information about its role in RA pathology and subsequently the relation to response to rituximab treatment. The three chapters of this part approached this issue from different perspectives, ranging from up- to downstream IFN signaling; whereas in **Chapter 3.1** the upstream activation of the IFN signaling pathway was studied, **Chapter 3.2** was focused on the identification of the cell type(s) in which this signaling would take place, and in **Chapter 3.3** we investigated the composition of the IFN response gene program itself.

In **Chapter 3.1**, we studied the ability of RA patient serum to induce a type I IFN response in healthy control peripheral blood mononuclear cells (PBMCs) and compared this to the well-characterized IRG-inducing ability of serum from SLE patients. This information could provide more insight into the source of induction of the type I IFN response in RA.

The exposure of healthy control PBMCs to patient sera revealed essential differences in IRG induction by RA and SLE serum. As previously described, serum from the majority of SLE patients caused a rapid IRG induction that appeared directly mediated by IFNα. Serum from RA patients, on the other hand, displayed IRG induction that was considerably slower (after 8 hours of incubation instead of 4 hours) which did not require IFNα activity but rather seemed to occur via another, indirect signaling route. This indicates that the source of the type I IFN response in RA is different from SLE. As expected, we observed that the IFN response induced by SLE serum

was associated to positivity for antinuclear antibodies. However, virtually all RA patients were negative for these antibodies, and their IRG induction was not correlated to positivity or titers of RA-related antibodies, such as rheumatoid factor or antibodies against citrullinated proteins either. Conclusively, we have demonstrated that serum from a part of the RA patients contains an IRG-inducing factor. Up until now, we know that the mechanism of IRG induction differs from that in SLE patients, but more detailed characterization requires follow-up experiments.

In **Chapter 3.2**, we investigated the contribution of the major peripheral blood leukocyte subsets to the type I IFN signature in peripheral blood from RA patients. This revealed that the signature is present in all subsets, but that it was most pronounced in the polymorphonuclear leukocyte, or granulocyte (PMN) fraction. This fraction displayed higher induction than $CD4^+$ T cells, $CD8^+$ T cells, monocytes and $CD19^+$ B cells, which could not be solely explained by the high abundance of PMNs in blood. Moreover, we observed elevated expression of the type I IFN receptors IFNAR1 and IFNAR2 in PMNs isolated from RA patients compared to PMNs isolated from healthy controls, whereas this was not the case for the RA PBMCs compared to healthy control PBMCs.

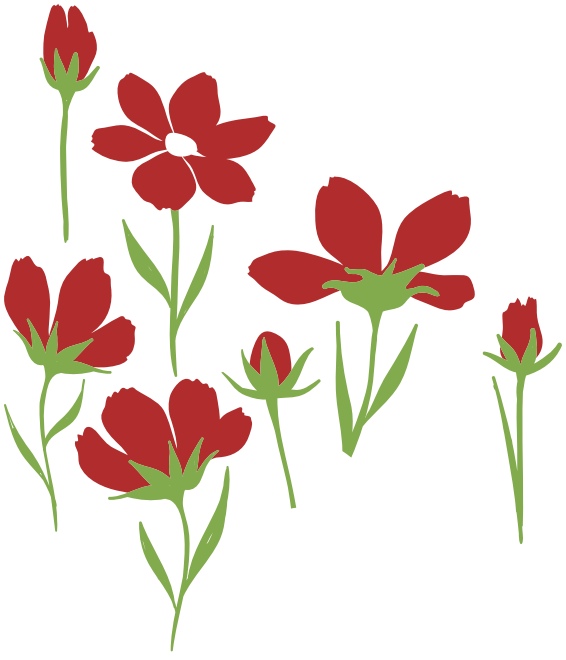
Altogether, these data indicate that RA PMNs are the main contributors to the IFN signature, which appeared to be caused by an increased sensitivity towards type I IFN signaling compared to other leukocyte subsets.

In **Chapter 3.3**, we studied the gene composition of type I IFN signatures in several autoimmune diseases. In systemic lupus erythematosus (SLE), it is known that the type I IFN signature is the result of predominant IFNα activity. Comparison of the IFNα-induced response gene profiles of SLE patients to those of IFNβ-treated MS patients resulted in the identification of gene profiles that reflect IFNα-dominant signatures (gene cluster (GC-) A) and IFNβ-dominant signatures (gene cluster (GC-) B). Using the log-ratio between these two profiles resulted in excellent separation of SLE patients and IFNβ-treated MS patients. These analyses were extended to myositis patients, IFNβ-naive MS patients and RA patients, which revealed that these diseases also displayed differences in the IFNα- and/or IFNβ dominance. All myositis patients showed a type I IFN response profile similar to the SLE patients, indicating IFNα dominance. MS patients, on the other hand, displayed large inter-individual variability; some patients displayed slight IFNα dominance, whereas others showed IFNβ dominance, or the GC-A/GC-B log-ratio approached zero, indicating similar contributions of both IFNα and IFNβ. RA patients displayed slight IFNα dominance in most patients, though less pronounced than SLE and myositis patients. The GC-A/GC-B log-ratios were closer to zero, which suggests involvement of both IFNα and IFNβ in RA. In conclusion, this study demonstrated that the IFN signatures display distinct differences between autoimmune diseases. Considering the pro-inflammatory nature of IFNα in SLE and the anti-inflammatory role of IFNβ in MS, specification of the type I IFN response in autoimmune diseases might give new insights into its role in pathology and/or its therapeutic potential.

In summary, the IFN signature is a biomarker for RA that shows potential clinical applicability provided that the interference by suppressive treatment is taken into account. Furthermore, the exact mechanism behind the IFN signature in RA remains to be unraveled, but we established that it appears different from SLE and myositis and mainly originated from peripheral blood granulocytes.

Appendix

Nederlandse samenvatting
List of abbreviations
List of publications
Curriculum Vitae
Dankwoord



Nederlandse samenvatting

Reumatoïde artritis

Reumatoïde artritis (RA) is een auto-immuunziekte die gekenmerkt wordt door chronische gewrichtsontstekingen. Door de reactie van het lichaamseigen immuunsysteem tegen de gewrichten ontstaat schade aan het kraakbeen en bot, welke leiden tot symptomen als gewrichtspijn en –zwelling. De ziekte treft ongeveer 1% van de wereldbevolking, waarvan twee keer zoveel vrouwen als mannen.

Op dit moment is de oorzaak van RA onbekend en kan het niet volledig genezen worden. RA staat bekend als een heterogene ziekte; hoewel alle patiënten dezelfde diagnose krijgen, bestaan er veel verschillen tussen individuen, bijvoorbeeld in de aantallen betrokken gewrichten, de mate van gewrichtsschade en de niveaus van ontstekingswaarden en antistoffen in het bloed. Als resultaat van deze heterogeniteit is er grote verscheidenheid in beschikbare therapieën en in de respons op elke therapie. Het verkrijgen van inzicht in de verschillen in cellulaire processen tussen patiënten en het vinden van voorspellers van respons op therapie is van essentieel belang om de ziekte beter te begrijpen en uiteindelijk therapie op maat mogelijk te maken.

Dit proefschrift

Er zijn reeds aanwijzingen dat de activiteit van type I interferon (IFN) een belangrijke rol speelt in RA en andere auto-immuunziekten. Type I IFNs zijn cytokines ("signaalstoffen") die een belangrijke rol spelen in het immuunsysteem; ze zijn aanwezig als reactie op virusinfecties, maar komen ook vaak voor in het bloed van patiënten met een auto-immuunziekte. Ongeveer 50% van de RA-patiënten heeft een zogenaamd "type I IFN-profiel" in het bloed, wat inhoudt dat ze verhoogde expressie vertonen van een groep genen die aangezet kunnen worden door type I IFNs, de type I IFN-respons genen (IRGs). Het is gebleken dat de aanwezigheid van dit IFN-profiel vóór de start van behandeling met het medicijn rituximab geassocieerd is met een slechte respons op deze therapie. Het onderzoek beschreven in dit proefschrift had als doel om meer te weten te komen over de klinische toepasbaarheid van dit IFN-profiel in RA en om inzicht te krijgen in de moleculaire mechanismen die dit profiel kunnen veroorzaken dan wel in stand houden.

Klinische karakterisering van het type I IFN-profiel in reumatoïde artritis

Het eerste deel van dit proefschrift, beschreven in **Hoofdstuk 2**, is toegespitst op de klinische toepasbaarheid van het IFN-profiel in RA.

Een belangrijke vraag die al heerst sinds de eerste beschrijving van het IFN-profiel in RA, is of deze gerelateerd zou zijn aan bepaalde klinische parameters. Deze kwestie is onderzocht in **Hoofdstuk 2.1**, waarin de mogelijke associaties zijn bestudeerd tussen de IRG-expressie in RA-patiënten en parameters zoals ziekteactiviteit, mate van ontsteking, aanwezigheid van gewrichtserosies en de aanwezigheid van autoantilichamen in het bloed. Dit leverde geen significante associaties op, hoewel we wel waarnamen dat de IRG-expressie onderdrukt werd onder behandeling met hydroxychloroquine (HCQ), sulfasalazine (SSZ) en/of prednison (PREDN). Ondanks deze observatie lijkt deze data erop te wijzen dat aanwezigheid van het IFN-profiel geen invloed heeft op de ernst van de ziekte.

De **Hoofdstukken 2.2** en **2.3** hadden als doel om het bestaande voorspelmodel met het IFN-profiel voor de respons op rituximab te optimaliseren. In **Hoofdstuk 2.2** is het effect van prednison op dit voorspelmodel bestudeerd. Het is eerder beschreven dat glucocorticoïden zoals prednison een onderdrukkend effect kunnen hebben op IFN-signalering. Aangezien er grote variatie is tussen RA-patiënten in het gebruik van prednison en de dosering, wilden we vaststellen wat de invloed was van prednisongebruik op het IFN-profiel en de daarmee samenhangende rituximab respons voorspelling.

In dit hoofdstuk tonen we nogmaals aan dat de IRG-expressie lager is in patiënten die prednison gebruiken ($PREDN^+$) vergeleken met patiënten die dit niet doen ($PREDN^-$). Daarnaast zien we dat deze verlaging het grootst is in patiënten die een hoge dosis prednison (≥10mg/dag) gebruiken. Als gevolg van deze verlaging blijkt ook het voorspelmodel op basis van het IFN-profiel minder goed te werken, doordat er meer overlap in IRG-expressie is tussen de responders en non-responders in de $PREDN^+$ groep vergeleken met de $PREDN^-$ groep. Van de $PREDN^-$ groep kan 92% van de patiënten correct geclassificeerd worden als responder of non-responder, terwijl dit slechts 63% van de $PREDN^+$ groep is. Dit wijst erop dat het belangrijk is om rekening te houden met prednisongebruik bij het gebruik van het voorspellende IFN-profiel.

Hoofdstuk 2.3 borduurt voort op de optimalisatie van de IFN-gerelateerde voorspelling van RTX non-respons. In de eerste plaats bevestigen we hier in twee nieuwe patiëntengroepen dat het IFN-profiel inderdaad voorspellend is voor de non-respons op rituximab. Ook zien we hier bevestigd dat prednisongebruik interfereert met het IFN-profiel en de voorspelling, al lijkt het effect niet even groot in beide groepen. Mogelijk is de termijn van prednisongebruik hierop van invloed. We presenteren een predictiemodel waarin het IFN-profiel gecombineerd wordt met klinische parameters die ook voorspellend zijn voor de respons op rituximab: de ziekteactiviteit (DAS28) vóór start van therapie, het gebruik van andere medicatie, en de positiviteit voor de autoantilichamen reumafactor (RF) en anti-citrulline antilichamen (ACPA). Combinatie van deze parameters resulteert in een voorspelmodel dat beter presteert dan elke variabele afzonderlijk. Wel lijkt de precieze combinatie en weging van variabelen afhankelijk te zijn van de prednison-status, dit zal verder onderzocht moeten worden.

Samengevat hebben we in **Hoofdstuk 2** van dit proefschrift laten zien dat hoewel het IFN-profiel geen specifieke ziektestaat lijkt aan te duiden, het mogelijk wel klinische toepasbaarheid heeft als voorspeller van non-respons op rituximab therapie. Om dit te bereiken is het van groot belang om IFN te combineren met andere voorspellende parameters en om vast te stellen wat het precieze effect van de prednison-gerelateerde IFN-suppressie is.

Moleculaire karakterisering van het type I IFN-profiel in reumatische aandoeningen

Naast de klinische toepasbaarheid van het IFN-profiel in RA, weten we ook nog weinig van de precieze mechanismen achter dit profiel. Het verkrijgen van meer inzicht hierin zou kunnen leiden tot beter begrip van de ziekte en de behandelopties. **Hoofdstuk 3** van dit proefschrift is gericht op deze kwestie, welke van verschillende kanten benaderd is; in **Hoofdstuk 3.1** proberen we meer te weten te komen over het proces dat vóór de activatie van het IFN-profiel plaatsvindt, in **Hoofdstuk 3.2** onderzoeken we de bijdrage van verschillende immuunceltypen aan het IFN-profiel en in **Hoofdstuk 3.3** bestuderen we de compositie van het IFN genprofiel zelf.

In **Hoofdstuk 3.1** is bestudeerd of serum van RA-patiënten in staat is om een type I IFN-respons aan te zetten in bloedcellen (PBMCs) van gezonde donoren. Dit zou kunnen duiden op de aanwezigheid van een activator van het IFN-profiel. Een soortgelijke studie is reeds beschreven voor serum van patiënten met systemische lupus erythematodes (SLE), wat dan ook is meegenomen als vergelijkingsmateriaal.

Uit de resultaten blijken er verschillen te zijn tussen het gedrag van RA serum en SLE serum. Het serum van de SLE-patiënten gedraagt zich zoals eerder beschreven: de meerderheid van de samples vertoont een snelle activatie (binnen 4 uur) van de IFN-respons in gezonde PBMCs, die geassocieerd blijkt te zijn met de aanwezigheid van IFNα eiwit. Serum van RA-patiënten vertoont echter pas later activatie van de IFN-respons, namelijk na 8 uur incubatie. De IFN-respons activatie door RA serum is niet afhankelijk van IFNα activiteit maar lijkt in plaats daarvan via een andere, indirecte signaleringsroute plaats te vinden. Deze route blijkt onafhankelijk van de aanwezigheid van antinucleaire antilichamen, welke een rol spelen bij de IFN-respons activatie door SLE serum, en RA-gerelateerde antilichamen zoals RF en ACPA.

Alles bij elkaar genomen hebben we laten zien dat serum van een deel van de RA-patiënten inderdaad een IFN-respons kan activeren, wat suggereert dat het een activerende factor bevat. Tot nu toe lijkt het mechanisme hierachter in ieder geval anders dan het mechanisme dat plaatsvindt in SLE-patiënten, maar vervolgexperimenten zijn noodzakelijk voor verdere karakterisering van de IFN-respons activatie en de activerende factor in RA serum.

Hoofdstuk 3.2 beschrijft onderzoek naar de bijdrage van de verschillende typen immuuncellen in het bloed aan het IFN-profiel. Het IFN-profiel wordt meestal bepaald in volbloed, wat bestaat uit verschillende celtypen; het is dus het resultaat van een combinatie van celtypen. In deze studie zijn de meest voorkomende typen immuuncellen geïsoleerd uit het bloed: T cellen, B cellen, monocyten en neutrofielen. Vervolgens is in zowel deze celsubsets als in het volbloed de IRG-expressie bepaald. Hoewel het IFN-profiel aanwezig blijkt te zijn in alle celtypen, is deze het meest prominent aanwezig in de neutrofielfractie. Neutrofielen hebben de grootste aantallen van alle celtypen in het volbloed, maar de hoge IRG-expressie in deze fractie blijkt niet alleen verklaard te kunnen worden door deze aantallen. Uit verder onderzoek blijkt dat de neutrofielen van alle bestudeerde celtypen de hoogste expressie vertonen van de receptoren waar type I IFNs aan binden (IFNAR), wat zou kunnen wijzen op een verhoogde gevoeligheid voor IFN-signalering. Dit wordt verder bevestigd door de waarneming dat de IFNAR expressie verhoogd is in neutrofielen van RA-patiënten vergeleken met neutrofielen van gezonde controles, maar niet in de overige immuuncellen.

Samengevat wijst deze data erop dat de neutrofielen van RA-patiënten de grootste bijdrage levert aan het IFN-profiel in bloed, mogelijk door een verhoogde gevoeligheid voor IFN-signalering.

In **Hoofdstuk 3.3** hebben we de compositie van genen in het IFN-profiel bestudeerd en vergeleken tussen verschillende auto-immuunziekten. Aanwezigheid van een IFN-profiel is namelijk niet uniek voor RA, maar ook beschreven voor SLE, multiple sclerose (MS) en myositis. Zoals hierboven reeds beschreven is het van SLE bekend dat het IFN-profiel voornamelijk wordt ver-

oorzaakt door de verhoogde aanwezigheid van het cytokine IFNα. Ook weten we reeds dat IFNα een pathogene rol speelt in SLE; het draagt bij aan het ontstaan van een vicieuze cirkel van ontstekingen. In MS daarentegen, is behandeling met het cytokine IFNβ, ook een type I IFN, heilzaam gebleken in een groot deel van de patiënten, hoewel dit niet het geval lijkt voor MS-patiënten die al een IFN-profiel hebben vóór behandeling. IFNα en IFNβ activeren in principe dezelfde signaleringsroute, maar blijkbaar kunnen ze zowel pro-inflammatoire als anti-inflammatoire functies uitoefenen. Om hier meer over te weten te komen hebben we de expressie van 23 IRGs, die door zowel IFNα als IFNβ aangezet kunnen worden, vergeleken tussen SLE-patiënten met een IFN-profiel en IFNβ-behandelde MS-patiënten. Het blijkt dat het verschil tussen deze "IFNα-profielen" en "IFNβ-profielen" ligt in de verhoudingen tussen de diverse genen. We identificeren een cluster met genen dat relatief hoger tot expressie komt in de SLE-patiënten ("IFNα-dominant", GC-A) en een cluster met genen dat juist relatief hoger tot expressie komt in de MS-patiënten ("IFNβ-dominant", GC-B). Door gebruik te maken van de log-ratio van deze twee clusters kunnen de SLE-patiënten vrijwel perfect onderscheiden worden van de IFNβ-behandelde MS-patiënten.

Vervolgens hebben we deze twee genclusters (GC-A en GC-B), ook bestudeerd in myositispatienten, IFNβ-onbehandelde MS-patiënten en in RA-patiënten, om meer te weten te komen over de IFNα- of IFNβ-dominantie in deze patiëntengroepen. De compositie van het IFN-profiel in myositispatiënten blijkt erg te lijken op die van de SLE-patiënten: er is een duidelijke GC-A-dominantie. Dit past ook met de parallellen tussen myositis en SLE die reeds bekend zijn, bijvoorbeeld in de typen autoantilichamen. De onbehandelde MS-patiënten vertonen behoorlijke verschillen tussen de patiënten. Er zijn een aantal patiënten die enige GC-A-dominantie vertonen, enkele patiënten die GC-B-dominantie vertonen, en een paar patiënten die een log-ratio dicht bij nul hebben, wat zou kunnen wijzen op betrokkenheid van zowel IFNα en IFNβ. De RA-patiënten vertonen lichte GC-A (dus waarschijnlijk IFNα-)dominantie, maar dit is lang niet zo hoog als gezien voor de SLE en myositispatiënten. De log-ratio's tussen GC-A en GC-B liggen dichter bij nul, wat ook zou kunnen betekenen dat er enige IFNβ bij betrokken is.

De conclusie die we kunnen trekken uit deze studie, is dat "het" IFN-profiel uitgesproken verschillen vertoont tussen auto-immuunziekten, en soms zelfs binnen een auto-immuunziekte. Afgaande op de pro-inflammatoire rol van IFNα in SLE en de anti-inflammatoire rol van IFNβ in MS, zou verder onderzoek naar dit soort verschillen nieuwe inzichten kunnen opleveren met betrekking tot de begrip van de ziekten en de behandelopties.

Conclusie

Samengevat laat dit proefschrift zien dat het IFN-profiel potentiële klinische toepasbaarheid heeft in RA, mits er rekening gehouden wordt met de interferentie door immunosuppressieve co-medicatie. Daarnaast is het precieze mechanisme achter het IFN-profiel nog steeds grotendeels onbekend, maar hebben we wel aanwijzingen dat de bron en het mechanisme van activatie verschilt van die in SLE en myositispatiënten, en dat de belangrijkste bron van het IFN-profiel in RA de neutrofielen zijn.

List of abbreviations

ACPA	Anti-Citrullinated Protein Antibody
ACR	American College of Rheumatology
ANA	Antinuclear Antibody
AUC	Area Under the Curve
CD	Cluster of Differentiation, indicates cell surface markers
CHX	Cycloheximide
CRP	C-Reactive Protein
DAS28	Disease Activity Score of 28 joints
DMARD	Disease-Modifying Anti-Rheumatic Drugs
ESR	Erythrocyte Sedimentation Rate
EULAR	European League Against Rheumatism
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase (housekeeping gene)
GAS	Gamma-Activated Sequence
GC	Gene Cluster (Chapter 3.3) or Glucocorticoid (all other chapters)
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HCQ	Hydroxychloroquine
IFN	Interferon
IFNAR	Interferon Alpha/Beta Receptor
IFNGR	Interferon Gamma Receptor
IIM	Idiopathic Inflammatory Myopathies, i.e. Myositis
IRF	Interferon Regulatory Factor
IRG	Interferon Response Gene
ISGF	Interferon-Stimulated Gene Factor
ISRE	Interferon-Stimulated Response Element
JAK	Janus Kinase
MS	Multiple Sclerosis
MTX	Methotrexate
NET	Neutrophil Extracellular Trap
NSAID	Non-Steroidal Anti-Inflammatory Drugs
PBMC	Peripheral Blood Mononuclear Cells
PMN	Polymorphonuclear Leukocyte, Granulocyte, Neutrophil
PREDN	Prednisone
PRR	Pattern Recognition Receptor
qPCR	Quantitative Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
ROC	Receiver Opererating Characteristics
RT-PCR	see qPCR

RTX	Rituximab
SD	Standard Deviation
SJC	Swollen Joint Count
SLE	Systemic Lupus Erythematosus
SSZ	Sulphasalazine
STAT	Signal Transducer and Activator of Transcription
TJC	Tender Joint Count
TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor Alpha
TYK	Tyrosine Kinase
VAS	Visual Analogue Scale

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* Authors contributed equally
‡ Shared last authorship

Curriculum Vitae

Tamarah de Jong werd geboren op 29 december 1985 te Den Helder. Daar groeide zij op en behaalde ze in 2004 haar VWO-diploma aan het Etty Hillesum College. In hetzelfde jaar startte zij met de bacheloropleiding Farmaceutische Wetenschappen en volgde aansluitend de masteropleiding Drug Discovery and Safety, beiden aan de Vrije Universiteit te Amsterdam. Tijdens de masteropleiding volgde ze de specialisatie Molecular Pharmacology en liep ze tweemaal stage. De eerste stage was bij de afdeling Medicinal Chemistry aan de VU, waar ze onderzoek deed naar de rol van de humaan cytomegalovirus-gerelateerde receptor US28 in pancreatitis, glioblastoma en darmkanker. De tweede stage was bij de Preclinical Candidate Selection unit van Solvay Pharmaceuticals te Weesp, waar ze werkte aan het opzetten van een assay om de activiteit van transglutaminase 2 de meten. Ze behaalde haar masterdiploma in 2009 en begon als analist bij Solvay Pharmaceuticals. In februari 2010 startte ze haar promotieonderzoek bij de Inflammatory Disease Profiling Unit onder leiding van Prof. dr. Cor Verweij aan de afdeling Pathologie van het VUmc. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Momenteel is ze werkzaam als postdoc bij de afdeling Reumatologie van het VUmc.

Tamarah de Jong was born on December 29th, 1985 in Den Helder. There, she grew up and finished her VWO at the Etty Hillesum College in 2004. The same year, she started her study Pharmaceutical Sciences, followed by the master's programme Drug Discovery and Safety, both at the VU University in Amsterdam. During her master's she followed a specialization track in Molecular Pharmacology and performed two internships. The first internship was at the department of Medicinal Chemistry at the VU, where she investigated the role of the human cytomegalovirus-related receptor US28 in pancreatitis, glioblastoma and colon cancer. Her second internship was at the Preclinical Candidate Selection unit of Solvay Pharmaceuticals in Weesp, where she worked on the development of an assay to measure transglutaminase 2 activity. She received her master's degree in 2009 and started working as a research technician at Solvay Pharmaceuticals. In February 2010 she started her PhD trajectory at the Inflammatory Disease Profiling Unit under supervision of Prof. dr. Cor Verweij at the Pathology department of the VU university medical center. The results of this research are described in this thesis. Currently, she is working as a postdoc at the Rheumatology department of the VUmc.

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Ook vrijwel elke woensdagavond staat gelijk aan een ontspanningsmomentje. Soms een bordspelletje, veel vaker een filmpje (als we eruit komen welke de meiden niet te eng vinden en Bram nog niet gezien heeft), of we praten gewoon en de avond gaat vanzelf voorbij. **Bram**, **Paul** & **Brenda**, **David** & **Ho-Yin**, bedankt voor jullie gezelschap! **David**, ook bedankt voor het mooi maken van dit boekje, ik ben er super blij mee!

Johan, onze maandelijkse maandag met zijn drieën is vrij spontaan ontstaan, maar volgens mij bevallen ze ons alle drie helemaal prima! Op naar de volgende :-)

Dan de ex-D&D-vriendengroep-en-aanhang: **Chris** & **Anna**, **Marcel** & **Maureen**, **Paul** & **Thirza**, **Sjoerd** & **Inge**, **Wollie** & **Madelon**, jullie zijn allemaal lieverds en maken elke verjaardag, etentje (al dan niet met veel te late diepgaande gesprekken), Pinkpop, Polderse Kermis, Strand van Luna-bezoekjes en Oud-en-Nieuw weer super gezellig!

Niet iedereen heeft een positieve associatie met het woord "schoonfamilie", maar ik heb toch echt niets te klagen, en dan heb ik ze nog wel dubbel! **Arie** & **Marian**, **Piet** & **Renny**, **Eline** & **Milo**, **Koen**, vanaf het begin heb ik me bij jullie welkom gevoeld. Ik hoop dat dit boekje een beetje duidelijk maakt waar ik de afgelopen jaren mee bezig ben geweest en wat soms best moeilijk was om uit te leggen.

Pap en **mam**, ik herinner me gesprekken tijdens de middelbare schooltijd over wat ik later wilde gaan doen, en wist (en weet) het eigenlijk nooit zo goed (de Jongkwaaltje?). Toch hebben jullie me altijd gesteund in zowel de keuze als de kosten en ik realiseer me dat dat geen vanzelfsprekendheid is. Bedankt!

Jessica & **Simon**, de frequentie van elkaar zien is misschien niet zo hoog, maar de kwaliteit eigenlijk wel altijd! Ik heb genoten van onze winterse vakanties (zullen we weer eens?) en alle andere ontmoetingsmomenten. **Jes**, als een echte grote zus ben je mij voorgegaan in het promotieproces en het onderzoek doen. Jij herkent en begrijpt veel van de perikelen en hebt altijd wel wijs advies voor me. Thanks zus! :-)

Broeders, **Arjan** en **Hinko**, het samen eten op de dinsdagavond werd al snel ook een ventilatie-moment voor het werk. Regelmatig een “en, weet je al iets over je artikel?” of een “wat doe je nou op zo’n dag?” conversatie (en toen dat laatste eenmaal opgehelderd was: “heb je lekker gepipet-teerd/geïncubeerd/geautoclaveerd?”) onder het genot van een bordje pasta/rijst/pannenkoek/ etc.. Het is passend dat jullie bij deze mijlpaal naast me komen staan, super leuk dat jullie mijn paranimfen willen zijn!

Wouter, allerliefste Wout, jij bent mijn stabiele basis, mijn thuis. Met elke overwinning juichte je mee en voor alle mindere momenten droeg je oplossingen aan (of ik daar nou op zat te wachten of niet ;-)). Je bent een meester in het relativeren en weet altijd een lichtpuntje te vinden op de momenten dat ik dat zelf even niet kan. Ik kan me niet voorstellen dat ik dit zonder jou zou doen, dus dat doe ik dan ook gewoon maar niet :-). Op naar de volgende 10 jaar (en meer)!♥

Diepe dalen, mooie paden
Ik glim bij wat ik zachtjes,
haast onhoorbaar, fluisterend zeg

Waar ik gelopen heb
Is van nu af aan een weg

(Acda & de Munnik – Vandaag ben ik gaan lopen)